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Characterization of *unc-119* homologues in the zebrafish, *Danio rerio*
by

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled "Characterization of *unc-119* homologues in the zebrafish, *Danio rerio*" submitted by Angela Gail Manning in partial fulfillment of the requirements for the degree of Master of Science in Molecular Biology and Genetics.

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Abstract

One of the major goals of biology is to understand the development and function of the nervous system. The study of the basic mechanisms underlying nervous system development and function in simpler organisms has been rewarding; genes first identified in model organisms such as *C. elegans* often have vertebrate counterparts with homologous functions in the nervous system.

In *C. elegans* the *unc-119* gene is expressed throughout the nervous system of both the embryo and adult and encodes a novel protein product (Maduro & Pilgrim, 1995). Due to its pan-neuronal expression it was thought that *unc-119* would play a basic role in the development of the nematode nervous system. Indeed, *unc-119* appears to be involved in a general aspect of neurite outgrowth since mutant animals display many abnormal axon projections. Interestingly, the mammalian homologues[1] of *unc-119*, HRG4 and RRG4 (human and rat retinal gene 4) are expressed after birth in maturing photoreceptor cells of the retina (Higashide *et al.*, 1996). The apparent restriction in expression of the *unc-119* homologues in vertebrates to specialized cells of the eye is surprising given the wide spread neuronal expression observed in the worm. The ability of HRG4 coding sequence to rescue all defects of *unc-119* mutant worms demonstrates that these genes are functionally interchangeable as well (Morris Maduro, pers. comm., September 1996; Maduro *et al.*, 1999). In addition, at the time this thesis project was begun (September, 1996) database searches revealed ESTs (expressed sequence tag) corresponding to HRG4, of fetal and non-retinal origins. Together these observations prompted us to investigate this gene family further. Specifically, we wished to determine whether this family of genes is expressed during development and in non-retinal tissues in vertebrates. To address these questions we chose to investigate the expression pattern of the *unc-119* homologue in the zebrafish. The zebrafish (*Danio rerio*) is a popular

vertebrate model system that combines the ease of genetic manipulation with embryological analyses.

In this thesis, results are described that suggest there are at least three *unc-119* homologues in the zebrafish genome. Two of these homologues (*unc119b* & *unc119c*) are expressed in non-retinal embryonic and adult tissues; the expression pattern of the third homologue, *unc119a*, has not been determined. These findings suggest that the *unc-119* gene homologues in mammals (e.g. HRG4 and RRG4) may have a more widespread expression pattern (e.g. extra-retinal) than previously thought.

[1] The term homologue is used to denote sequence similarity at the nucleotide or protein level without proposing specific evolutionary relationships.

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Introduction

General Introduction

One major goal in biological research is to understand how the nervous system develops and functions. Considering the complexity of human physiology this task may seem unattainable, however studies in simpler organisms have aided us in understanding the basic mechanisms of this phenomenon. The transparency, small nervous system and genetic tractability of the invertebrate nematode *C. elegans* are a few of the features that have made it a favored organism for such studies (Brenner, 1974; Riddle *et al.*1997). Many genes with roles in nervous system development and function discovered in *C. elegans* have been found to have homologues in vertebrate species. For example, many molecules involved in axon guidance, such as UNC-6 and UNC-40, have vertebrate homologues with identical functions (reviewed in Culotti & Merz, 1998).

unc-119 is a *C. elegans* gene that has been studied extensively in our laboratory (Maduro & Pilgrim, 1995; Maduro *et al.*, 1999; Materi *et al.*, submitted). *unc-119* is expressed throughout the nervous system of embryos and adults and *unc-119* mutant worms display abnormal axon outgrowth and various behavioral defects. These observations suggest that *unc-119* is important for the proper development and function of the nematode nervous system.

Interestingly, rat and human genes (RRG4 and HRG4, respectively) that share a high degree of sequence similarity with *unc-119* were originally identified as having retinal specific expression (Higashide *et al.*, 1996). Since the HRG4 coding region (coupled with the regulatory elements of the *unc-119* gene) can fully rescue *unc-119* mutant defects this demonstrates that the human protein can perform the same biochemical function (as yet unknown) as UNC-119 in the worm (Morris Maduro, pers. comm., September 1996; Maduro *et al.*, 1999). At the time that the work in this thesis

was initiated (September, 1996) there existed only indirect evidence that vertebrate *unc-119* homologues are expressed embryonically and in non-retinal tissues (e.g. EST database searches revealed the presence of HRG4 sequences in libraries prepared from non-retinal & fetal tissues). Recently, direct experimental evidence that HRG4 is expressed in non-retinal tissues has been published (Swanson *et al.*, 1998), but these observations have not been independently confirmed.

The original observations suggested that vertebrate *unc-119* homologues could function earlier in development and perhaps in non-retinal tissue. Since these possibilities had not been addressed we decided to examine the *unc-119* homologue and its expression pattern in the zebrafish, *Danio rerio*. The zebrafish was chosen since it offers many advantages over other vertebrate model systems; primarily the ease of studying embryonic expression. Degenerate oligonucleotide primed PCR, RT-PCR, cDNA analysis, Northern analysis and whole-mount *in situ* hybridization techniques were used to analyze the temporal and spatial expression of the zebrafish *unc-119* homologue.

The results obtained suggest that there are at least three *unc-119* homologues in the zebrafish genome. Two of these homologues, *unc119b* and *unc119c*, are strongly expressed in the embryonic zebrafish brain and developing fin bud. Lower level expression of *unc119b* and *unc119c* is detected in other tissues of the zebrafish embryo (presumably neural). *unc119b* and *unc119c* are also expressed in non-retinal tissues of the adult. Since the zebrafish is an excellent system in which neuron development, function and behavior can be correlated (Fetcho & Liu, 1998) it will be interesting to continue the study of these genes. Future studies of the zebrafish *unc119* genes could contribute to our understanding of how the nervous system develops and functions in vertebrates.

C. elegans : a model for studies of the nervous system

The nematode, *C. elegans*, was first chosen as a suitable model for studies of the nervous system by Sidney Brenner (1974). This small worm offers many features that make it amenable to such analyses. The complete development of the worm can be observed in real time due to its transparency. The development of the worm follows a stereotyped pattern such that the positions and number of cells is invariant from animal to animal (Sulston and Horvitz, 1977; Sulston *et al.*, 1983). The animals are small (~1000 cells); the nervous system comprises approximately 30% of these cells. This manageable number of cells and invariant lineage has allowed researchers to completely describe the placement and connectivity of all the cells of the nervous system (White *et al.*, 1986). This achievement suggests that it may be possible to one day understand how specific behaviors are generated by the interactions between the neurons of the animal.

A large number of mutations affecting various aspects of nervous system development have been studied in the worm and have been reviewed in detail elsewhere (Ruvkun, 1997; Antebi *et al.*, 1997). Many of these mutants were first identified by a lack of movement or uncoordinated (unc) phenotype; further studies revealed defects at many levels of development and function of the nervous system. These analyses allowed researchers to establish the primary events involved in nervous system development including specification of neural fate, differentiation of specific neural cell types and axon outgrowth. I will briefly discuss some of the genes involved in these processes in *C. elegans*.

As mentioned above, an invariant pattern of cell division is responsible for giving rise to all the cells of the worm. It has been observed that certain patterns of cell divisions correlate with the production of specific neural types suggesting that lineage and cell specification are related genetically (Ruvkun, 1997). Many mutants that disrupt

the specification of cell fate have been identified. Hierarchies of transcription factors appear to be involved in the initial stages of determination of cells that will become neuronal. Although cell lineage plays a key role in specifying neural fate this information is modified by the expression of HOM-C genes in different regions along the anterior-posterior axis of the developing animal (Ruvkun, 1997). Reiterated cell lineages in the anterior and posterior regions of the animal may give rise to different neuron types or cell fates due to the action of different HOM-C genes in these regions and their combinatorial affects with each other and downstream lineage specific factors. The genes *mab-5*, *lin-39*, and *egl-5* are three members of the HOM-C cluster that have been studied in detail; these genes have homologues in Drosophila and vertebrates (e.g. homeobox genes). The homeobox genes of higher metazoans have undergone expansion and their interactions are more complicated overall; a necessity to direct the development of increasingly complex body patterns. The homeobox genes of higher metazoans also function to provide positional information and transmit this information to downstream genes, however pathways leading from specific homeobox gene expression to specification of neuron type in vertebrates is not as clear as in *C. elegans*. An example of the actions of the HOM-C genes in *C. elegans* would be the modification of the fates of identical reiterated cell lineages. For example, a subset of one particular cell lineage (the P cell lineage) gives rise to neurons required for vulval muscle function, while the remaining cells alternatively undergo programmed cell death. These alternate fates are a result of the differential action of HOM-C genes in various regions of the animal (Ruvkun, 1997). The HOM-C genes are believed to act through modifying the response of downstream neural specification genes (Ruvkun, 1997).

Specification of neural fates depends on a hierarchy of transcription factors. An example is the differentiation of mechanosensory neurons that illustrates how neural specification is achieved. The product of the *lin-32* gene is a transcription factor of the basic helix-loop-helix (bHLH) type that is necessary and sufficient for neuroblast

specification (Zhao and Emmons, 1995). In the absence of *lin-32* function, cells that would normally adopt a neuronal fate instead become hypodermal seam cells (Ruvkun, 1997). In a subset of neuroblasts LIN-32 (in conjunction with other factors; e.g. *vab-3* product) appears to be responsible for stimulating the expression of *unc-86* (Ruvkun, 1997). *unc-86* encodes a homeodomain containing transcription factor that appears to be required for maintaining the asymmetry between mother and daughter cells in certain neuroblast lineages (Chalfie *et al.*, 1981). UNC-86 is necessary for the expression of the *mec-3* gene (Xue *et al.*, 1992) which encodes yet another transcription factor. MEC-3 is thought to control the expression of the *mec-4* and *mec-7* genes that produce structural proteins unique to mechanosensory neurons (Mitani *et al.*, 1993).

In conjunction with or after differentiation, neuronal cells must interconnect to form a functioning nervous system. To achieve this axon outgrowth must occur. The guidance system involved in axon outgrowth is extremely complex and combines the action of secreted molecules, receptors, cytoskeletal components and transcription factors that regulate the expression of appropriate genes. UNC-6 is an example of an axon guidance molecule; it is highly related to the vertebrate netrin proteins (Ishii *et al.*, 1992; reviewed in Culotti & Merz, 1998). UNC-6 is not thought to be inherently attractive or repulsive to axons, rather its presence is interpreted as attractive or repulsive based on the properties of the cell that encounters it during migration (Ruvkun, 1997). UNC-40 has been implicated as a possible interpreter of UNC-6 signals, and since UNC-40 is a transmembrane protein it could function in coupling the detection of UNC-6 in the extracellular environment with rearrangements in the cytoskeletal machinery (Keino-Masu *et al.*, 1996). A protein with a definitive role in coupling extracellular guidance cues with cytoskeletal rearrangements that affect axon growth or retraction is UNC-44. UNC-44 is an ankyrin protein that functions by linking actin and spectrin filaments to various membrane proteins (Otsuka *et al.*, 1995).

The brief review above does little to address the real complexity of the nematode nervous system, however it does establish the usefulness of this system and its applicability to more complex organisms. Specifically, at every level of development from cell specification to axon guidance, homologues of key proteins have been found in other metazoans where they perform similar functions. Additionally, the studies conducted in the comparatively simple nematode have made it possible to more clearly understand the molecular actions of these genes.

Although the vertebrate nervous system arises through inductive interactions between tissues rather than modification of reiterated cell lineages as in *C. elegans*, many of the genes involved in the control of cell specification are homologous and function in similar pathways. Transcription factors of the bHLH type function in establishing areas where neuroblasts will arise in both insects and vertebrates (Arendt & Nubler-Jung, 1999). In Drosophila, *atonal* and *achaete/scute* represent homologues of *lin-32* and in vertebrates, *neurogenin*, has been identified (Jarman *et al.*, 1995; Arendt & Nubler-Jung, 1999). Furthermore, the POU homeodomain transcription factor UNC-86 has been conserved in vertebrates (e.g. human, mouse, cat and monkey) which contain three homologues Brn-3a, Brn-3b and Brn-3c that are expressed in sensory neurons including the retinal ganglion cells (Xiang *et al.*, 1995). *vab-3* appears to act upstream of *unc-86* in the nematode (Baumeister *et al.*, 1996); *Pax-6*, a vertebrate *vab-3* homologue, may act upstream of the Brn-3 genes (Xiang *et al.*, 1993). Therefore, homology is not conserved merely between genes involved in worm and vertebrate nervous system development, but in some cases entire pathways are conserved as well.

Many of the proteins responsible for axon guidance are conserved among metazoans. UNC-6 is related to the netrin proteins found in vertebrates, both molecules are expressed in the ventral most region of the developing nervous system where they function to guide axon growth (Kennedy *et al.*, 1994; Serafini *et al.*, 1994). Also the putative receptors for these secreted molecules, UNC-40 and DCC, respectively, are

homologous to each other (Chan *et al.*, 1996; reviewed in Culotti & Merz, 1998). It is apparent that studies of the nervous system of the worm have been, and will continue to be, extremely helpful in understanding the basic mechanisms underlying the development of the nervous system in all metazoans.

The analysis of the *unc-119* gene, which is expressed throughout the nervous system of the worm, was initiated with the hope that it might also have homologues in more complex organisms with a conserved role in nervous system development or function. When mammalian homologues of *unc-119* were found with an apparent restriction of expression to the retina in post-natal stages, we wished to explore the possibility that these homologues were expressed earlier in development and in non-retinal tissues of vertebrates. Since these issues had not been addressed we chose to characterize the expression pattern of the zebrafish *unc-119* homologue. Prior to introducing the data obtained in the zebrafish study I will first describe what is known about the *unc-119* gene in *C. elegans* and its mammalian homologues, HRG4 and RRG4.

unc-119 in *C. elegans*

unc-119 was first identified as a gene required for normal nervous system development and function in *C. elegans* (Maduro & Pilgrim, 1995). The most notable defect in homozygous mutant worms is a lack of movement. *unc-119* mutants are defective in sensing environmental cues and are thus unable to enter an alternative, protected larval state (dauer) under the appropriate conditions. A weak egg-laying defect is also observed in mutant animals.

X-gal staining of animals carrying *unc-119*::lac-Z fusion constructs demonstrates that *unc-119* is expressed throughout the nervous system beginning in the early embryo (in presumptive neurons) and this expression is maintained into the adult. Observations

of *unc-119* mutant worms expressing an *unc-119*::GFP (Green Fluorescent Protein) fusion construct allows the visualization of various morphological defects in mutants, including abnormal axon outgrowth (Maduro & Pilgrim, 1995). GFP fusion constructs expressed in subsets of neurons (Materi *et al.*, submitted) have allowed finer analysis of the morphological defects in *unc-119* mutants. These studies allow observation of multiple defects in axon outgrowth in specific neurons and defasciculation of the ventral nerve cord (VNC). Cell bodies in the VNC normally send axon projections (commissures) to the dorsal nerve cord in a straight, unbranched fashion (**Figure 1A & 1B**). In *unc-119* mutants these commissures are often highly branched and may terminate without reaching their target (Materi *et al.*, submitted) (**Figure 1C**). The movement defect of *unc-119* mutants can be explained by these morphological observations. Movement is dependent on the coordinated activities of the ventral and dorsal nerve cords and the formation of proper synaptic connections to muscle cells; if these connections are disrupted normal movement cannot occur.

In wild-type animals, amphids (chemosensory neurons in the head of the animal) are exposed to the environment and function to receive information from the surroundings in the form of chemical cues. In *unc-119* mutants the amphids are shortened and possibly not exposed to the environment (Materi *et al.*, submitted). The axons of these chemosensory neurons are often abnormally branched or terminate prematurely potentially destroying their ability to form correct synapses with other cells (Materi *et al.*, submitted). These morphological defects can account for the inability of *unc-119* mutants to respond appropriately to changes in environmental conditions. Wild-type animals will enter an alternative larval form (dauer) under conditions of low food and high pheromone levels. *unc-119* mutants are unable to enter the dauer state (Maduro & Pilgrim, 1995). The defect lies in the inability of the mutants to sense chemical cues in their environment. However, the animals are capable of undergoing the physiological

changes required to enter the dauer state; in combination with the *daf-7* mutation (a dauer constitutive mutant) *unc-119* animals do enter the dauer state (Maduro & Pilgrim, 1995).

unc-119 mutants retain eggs and produce broods approximately 50% the size produced by wild-type animals (Maduro & Pilgrim, 1995). In addition, unlike wild-type animals, *unc-119* mutants will lay eggs in the absence of food (Maduro & Pilgrim, 1995), suggesting that the regulation of egg-laying behavior has been disrupted. This phenotype may arise from a functional or structural abnormality in the hermaphrodite specific neurons (HSNs), which are involved in egg-laying (Materi *et al.*, submitted).

When *unc-119* was initially cloned and sequenced (Maduro & Pilgrim, 1995) it was novel, sharing no significant similarity to other sequences in GenBank. *unc-119* shares very weak sequence similarity (~20% identity) with a predicted *C. elegans* gene, C27H5.1, but this will not be discussed further. In the next section I will review what is known regarding the mammalian homologues of *unc-119* that were reported after the initial *unc-119* publication (Maduro & Pilgrim, 1995; Higashide *et al.*, 1996).

HRG4/RRG4 – mammalian homologues of *unc-119*

Shortly after the initial publication describing *unc-119* (Maduro & Pilgrim, 1995), sequences with similarity to the nematode gene were reported (Higashide *et al.*, 1996). HRG4 and RRG4 represent mammalian homologues of *unc-119*. The overall identity of UNC-119 with these proteins is approximately 50%. The conservation between proteins is unevenly distributed; the carboxyl termini are much more highly conserved than the amino termini (**Figure 2**). HRG4 and RRG4 are only 67% identical in the first 50-60 amino acids (240 amino acids in total), but are 100% identical for the remainder of the protein (Higashide *et al.*, 1996). Recently, an additional human *unc-119* homologue has been discovered in the EST database (Dave Pilgrim, pers. comm., 1999); the conceptual amino acid sequence is also shown in **Figure 2**.

HRG4 and RRG4 were originally identified by Higashide *et al.* (1996) through a subtractive library screening approach designed to identify genes with retinal enriched expression. Their Northern analyses reveal that the human and rat transcripts are highly expressed in the retina. They demonstrate that RRG4 is significantly expressed beginning at PND 5 (post-natal day 5), coinciding with the onset of photoreceptor cell differentiation in the rat retina. This expression continues to increase to PND 23, when the retina is fully developed. After this time the expression level plateaus and is maintained in the adult. HRG4 and RRG4 transcripts localize to the photoreceptor cells of the retina as assayed by *in situ* hybridization experiments (Higashide *et al.*, 1996). Production of an RRG4 antibody and use of indirect immunofluorescence and electron immunohistochemistry allowed this group to observe that the protein localizes specifically to rod and cone photoreceptor cell axons and synapses (Higashide *et al.*, 1998). The authors note that this localization pattern is reminiscent of proteins involved in synaptic vesicle recycling, and propose that the proteins may function in some aspect of neurotransmission.

The work of another group (Swanson *et al.*, 1998) corroborates the localization of the RG4s message to the photoreceptor cell layer of the retina. An interesting feature of HRG4 was revealed by the work of Swanson and colleagues. HRG4 produces alternate splice forms that are detected at different frequencies in a human retinal cDNA library that they screened. A 1.4 kilobase cDNA (82% frequency) and a 1.7 kilobase cDNA (18% frequency) were identified. The transcripts differ at their 3' ends; the 1.7 kilobase form retains the fourth intron that contains an in-frame stop codon. This would translate into different carboxyl termini for the two proteins. The 1.7 kilobase cDNA would produce a protein that replaces the last 37 amino acids coded for by the 1.4 kilobase cDNA with 17 new amino acids. Through Northern analysis and RT-PCR experiments Swanson and colleagues show that the transcripts differ in their expression pattern. The 1.4 kilobase transcript is highly expressed in the retina with low levels of expression in

other tissues such as adrenal gland, kidney and cerebellum. The 1.7 kilobase transcript is detected only in the retina, but at a much lower level than the 1.4 kilobase transcript. Whether the alternately spliced form represents a biologically significant transcript remains to be determined. Interestingly, both cDNAs are detected in a bovine cDNA library whereas only the more frequent cDNA is detected in a mouse library (Swanson *et al.*, 1998). The expression data obtained by the Swanson group suggest a possible role for HRG4 in tissues other than the retina (Swanson *et al.*, 1998).

The multiple defects of *unc-119* mutant worms can be rescued by injection of HRG4 cDNA (corresponding to the 1.4 kb transcript) flanked with 5' and 3' non-coding regions of the *unc-119* gene of *C. elegans* (Maduro *et al.*, 1999). This rescue is abolished when the hybrid construct is deleted for 10 highly conserved carboxyl terminal amino acids (Maduro *et al.*, 1999). The poorly conserved amino terminus (first 60 amino acids) is not required for rescuing function. It would be interesting to determine whether the 1.7 kilobase (retina specific) HRG4 cDNA is capable of rescuing any of the *unc-119* defects in light of the importance of particular carboxyl terminal amino acids for rescue. The observation that HRG4 can replace UNC-119 function in the worm demonstrates that the two proteins can perform the same biochemical function.

The mammalian homologues of *unc-119* have apparently high levels of expression only in the photoreceptor cells of the retina after birth; this contrasts with the expression pattern of *unc-119* throughout the nervous system of *C. elegans* in both embryos and adult worms. Another invertebrate, Drosophila, also possesses an *unc-119* homologue (*Dmunc-119*) that is expressed in the developing central nervous system and likely, in adult flies (Maduro *et al.*, 1999). These results suggested that mammalian *unc-119* homologues could be expressed in non-retinal tissues in embryonic and adult stages. Since these possibilities had not been addressed we decided to analyze the expression pattern of the *unc-119* homologue in the zebrafish, which has become a useful vertebrate model system. I will next focus on the advantages that make zebrafish so favorable. In

the following section I will describe the development of the nervous system in vertebrates noting where the zebrafish differs from the "standard" mode of development. This comparison strives to establish that the zebrafish is "general" enough to be useful for nervous system studies and that information gained in the zebrafish will have applicability for other vertebrate systems. I will then describe features that make the zebrafish particularly well suited for studies of the nervous system. Finally I will present and discuss results obtained in the analysis of the *unc-119* homologues in the zebrafish.

The zebrafish as a vertebrate model system

Studies of developmentally important genes in invertebrates such as *C. elegans* and Drosophila and their homologues in vertebrate systems have established that many important pathways in development are conserved between the two phyla (e.g. signals involved in establishing the dorsal/ventral axis). These studies have been useful, however they suffer from the limitation that invertebrates do not possess analogs of certain structures present in vertebrates (e.g. neural crest) (Ingham, 1997). In addition, similar structures present in invertebrates may have only superficial similarities to the same structure present in vertebrates as a result of convergent evolution (e.g. the eye of Drosophila).

In the early 1980's George Streisinger at the University of Oregon began developing the zebrafish as a model organism. Streisinger and colleagues addressed the difficulty in studying recessive mutations in vertebrate organisms by developing methods to uncover recessive phenotypes in one step in the zebrafish (Streisinger *et al.*, 1981 & described in Westerfield, 1995). They found that haploid embryos could be obtained through the fertilization of eggs with sperm whose genome has been inactivated by ultraviolet light. Homozygous diploids could be obtained by treating haploids with early

pressure or heat shock to inhibit early divisions. The zebrafish offers this and many other advantages over other vertebrate model systems (reviewed in Kimmel, 1989; Westerfield, 1995;Granato & Nusslein-Volhard, 1996; Schier *et al.*, 1996; Postlethwait & Talbot, 1997).

Zebrafish are small, hardy fish that are inexpensive to maintain. When adults are kept under an artificial light cycle, females will produce hundreds of transparent embryos that develop synchronously and external to the mother. These two features allow direct observation of developmental events in the living animal. Development of the zebrafish occurs rapidly, within 12 hpf (hours post fertilization) the basic body plan characteristic of all vertebrates has been established (**Figure 3**). The muscle and nervous systems begin functioning by 17 hpf and the heart and notochord are easily observed by 24 hpf (**Figure 3**). By 5 dpf (days post fertilization) major organ systems are visible and functioning (**Figure 3**). The fish have a relatively short generation time and reach sexual maturity at three months. The reproductive life span is approximately two years.

The genome size of the zebrafish is comparable to that of other vertebrates; 1.7 X 10^9 base pairs and the diploid genome is composed of fifty metacentric chromosomes (Postlethwait & Talbot, 1997; Zon, 1999). Presently, over 1500 markers and 400 cloned genes have been assigned positions on the genetic map; the average interval between markers is 1500 kilobases (compared to 700 kilobases on the human map) (ref. 36 in Postlethwait and Talbot, 1997). Many techniques are being employed to increase the density of markers and place additional zebrafish genes on the map (Schier *et al.*, 1996; Postlethwait & Talbot, 1997; Zon, 1999).

The above features make the zebrafish an excellent system for embryological studies and for genetic screens aimed at identifying developmentally important genes. The *in utero* development of the mouse, the classical genetic model for vertebrates, severely restricts this organism as a choice for a large- scale mutagenesis screen. However, the mouse still has some advantages over zebrafish. For example, embryonic

stem (ES) cell cultures and gene targeting techniques have not been successfully developed in the zebrafish (Driever *et al.*, 1994).

The journal Development dedicated a complete volume to the description of many developmentally important genes uncovered in large-scale screens carried out recently in the zebrafish (Driever *et.al*, 1996; Haffter *et.al*, 1996). Hundreds of genes involved in specific developmental pathways in the zebrafish were identified and continue to be analyzed. Many of the mutants identified phenocopy human diseases such as cardiovascular defects and blood disorders (Grunwald, 1996; Zon, 1999). This observation, coupled with the discovery that zebrafish and human genomes share large regions of synteny, is exciting since it suggests that mapping and sequence information obtained from one organism can be readily applied to the other. For example, zebrafish with mutations resembling human disorders can be studied to uncover the molecular basis of the defect. Once a map position is assigned for the zebrafish mutation this information can be used to find a human candidate gene for the disorder (if the zebrafish mutation maps to a region shared between the two genomes) (Postlethwait *et.al*, 1998 and Zon, 1999).

One feature of the zebrafish genome was first thought to pose a severe drawback in its use as a model. It appears that a partial or complete duplication of the genome occurred after the divergence of the zebrafish from the mammalian lineage (Amores *et al.*, 1998; Postlethwait *et .al*, 1998; reviewed in Meyer & Malaga-Trillo, 1999). It was thought that analysis of gene function could be perturbed by redundant or overlapping functions of duplicated genes. However, in some cases, this duplication event may prove useful in analyzing gene function. For example, a single *engrailed-1* gene in mammals is expressed in both the developing hindbrain and limb buds. In zebrafish there are two *engrailed* genes that are homologues of *engrailed-1* in mammals; *eng1b* is expressed in the developing hindbrain and *eng1* is expressed in the developing fin bud (Force *et al.*, 1999; reviewed in Vogel, 1998). If a single mammalian gene is performing two or more

functions during development it may be difficult to study since knocking the gene out at an early stage may be lethal thereby masking its later function(s). In zebrafish a single mammalian gene may have two or more counterparts each performing one function. This type of arrangement could facilitate the study of the mammalian gene since each zebrafish homologue can be mutated without perturbing other pathways that members of the gene family may be involved in (Detrich *et al.*, 1999b).

The zebrafish is proving to be amenable to many techniques required for establishing a complete understanding of gene function in one organism. Xenopus is an excellent system for embryological studies (e.g. tissue transplantation), but is difficult to work with genetically. Mice have traditionally been used for genetic analysis, but *in utero* development has prevented observation of events occurring in real time. The zebrafish combines the ability of single cell transplantation and genetic analysis in one organism as well as offering researchers an opportunity to view developmental events (normal or disrupted by mutation) as they occur. In the next section I will describe the development of the nervous system of vertebrates and establish that the zebrafish is "general" enough to serve as a model system for these studies. I will then focus on further advantages of the zebrafish particularly as they pertain to studies of the development of the nervous system and its function.

Vertebrate Nervous System Development

When embarking upon the use of an organism for studies that will involve comparative aspects it is important to understand where differences in development and function of the relevant systems lie between the chosen model and other organisms. How generalized is the development of the nervous system of the zebrafish and how applicable will the information gained in studies of its nervous system be for other vertebrate

systems? I will review what is known regarding the major morphological events of vertebrate nervous system development, as well as some of the molecules involved, where they are known, addressing where zebrafish development differs from that of other vertebrates. At a very gross level the development of the nervous system of any vertebrate involves the same basic events. Early patterning of the embryo lays the groundwork for the formation of three primary germ layers and inductive interactions between prospective germ layers establish neural identity in ectodermal tissue. The neural anlage is then patterned along the anterior-posterior axis into the brain and spinal cord; concurrently neurons differentiate, undergo axon outgrowth and establish synpatic connections leading to a functioning nervous system. Despite some of the differences in developmental style that will become apparent below, it is important to note that fate maps constructed at the onset of gastrulation for the zebrafish (Kimmel *et al.*, 1990) demonstrate that the zebrafish embryo is organized similarly to other vertebrate embryos.

Formation of the Organizer

The earliest events in nervous system development involve the formation of an organizer, which functions to establish spatial patterning in the embryo by instructing surrounding cells to take on new fates (Ruiz i Altaba, 1998). Spemann and Mangold first identified the organizer in Xenopus in the early 1920's ("Spemann Organizer") and it has been extensively studied since (Spemann & Mangold, 1924). Equivalent structures have been identified in other vertebrate species through transplantation experiments (summarized in Solnica-Krezel *et al.*, 1996). The embryonic shield in the zebrafish possesses all of the inductive properties expected of the organizer (e.g. induction of a secondary body axis) (Oppenheimer, 1936; Ho, 1992).

In Xenopus the organizer is thought to be partly induced through signals derived from vegetal pole cells destined to form endoderm whereas the zebrafish embryonic

shield appears to arise as a result of signals emanating from the yolk syncytial layer, which underlies the zebrafish blastoderm (Kimmel *et al.*, 1995). This alternate source of inductive signals results from a difference between the two embryos; the early cells of the zebrafish embryo do not undergo complete cleavage and therefore, remain in contact with the vegetally positioned yolk cell (reviewed in Schier & Talbot, 1998). The second set of signals involved in organizer formation come from the Nieuwkoop centre in Xenopus, which is composed of dorsal vegetal cells of the blastula. In contrast, the dorsal yolk syncytial layer appears to provide the second set of signals in zebrafish (reviewed in Schier & Talbot, 1998). Nonetheless, these signals result in the specification of cells in the equatorial region of both embryos to ultimately become mesoderm during gastrulation (Schier & Talbot, 1998). In zebrafish the development of the embryo atop the yolk cell makes it more difficult to distinguish mesoderm and endoderm layers from each other in the gastrula stage than in other vertebrates and therefore, it is often referred to as mesoendoderm instead (Kimmel *et al.*, 1995). Regardless of the differing sources of signals required to induce organizer formation in Xenopus and zebrafish embryos, the signals themselves appear to be conserved between the two species; organizer formation appears to involve TGF-β related molecules and components of Wnt signalling pathways (Schier & Talbot, 1998).

Functions of the organizer

Once established the organizer goes on to pattern mesoderm dorsal-ventrally and induces the formation of the nervous system from ectoderm (reviewed in Schier & Talbot, 1998). The organizer appears to function in dorsal-ventral patterning by producing factors that nullify the effects of ventralizing signals such as BMP-4 and BMP-2 (bone morphogenetic protein) by binding them directly. Ultimately, gradients of these signals function to specify positional information along the dorsal-ventral axis in Xenopus (reviewed Schier & Talbot, 1998). In the zebrafish mutant screens have

identified genes important for dorsal-ventral patterning such as *swirl*, which encodes BMP2b, a member of the BMP family (see refs 26-28 in Schier & Talbot, 1998).

Many of the molecules with a role in dorsal-ventral patterning of the mesoderm also appear to function in inducing neural fates in the appropriate cells. In Xenopus the secreted protein chordin appears to be involved in binding and disrupting the ventralizing activity of BMP2 and BMP4; this relationship is detected genetically between the zebrafish *chordino* and *swirl* genes (reviewed Schier & Talbot, 1998). These antagonistic interactions appear to be required to block the induction of epidermal rather than neural fates in ectoderm (reviewed in Schier & Talbot, 1998).

Additional sources of patterning signals

In Xenopus, zebrafish, and potentially, other vertebrates the classical organizer discussed above may not be the only major source of patterning information required for formation of the nervous system in the embryo (reviewed in Ruiz i Altaba, 1998 and Schier & Talbot, 1998). In particular transplantation experiments involving the organizer of Xenopus or embryonic shield of the zebrafish have demonstrated that anterior-posterior patterning of the neural axis may involve signals other than those provided by the classical organizer (reviewed in Ruiz i Altaba and Schier & Talbot, 1998). In the zebrafish a newly discovered anterior organizer located in the ectoderm can direct surrounding cells to adopt the fate of anterior forebrain when transplanted to more posterior positions (reviewed in Ruiz i Altaba, 1998). The zebrafish ANB organizer (anterior neural boundary organizer) appears to have counterparts in other vertebrate species although the precise details have not been worked out as yet (reviewed in Ruiz i Altaba, 1998).

Neural tube formation

The morphogenesis of the neural anlage in zebrafish is quite different from other vertebrate species. Primary neurulation describes the mechanism by which the folds of the neural plate of most vertebrates migrate toward the midline resulting in the formation of a hollow neural tube. In contrast the neural tube of the zebrafish arises through secondary neurulation in which the neural plate folds into a solid neural keel which later undergoes cavitation to form a hollow neural tube (Kimmel *et al.*, 1995). Although the morphogenetic movements overall are quite different, the two processes are equivalent at the cellular level as ascertained through fate-mapping studies (Kimmel *et al.*, 1990; Papan & Campos-Ortega, 1994).

Brain Morphogenesis and Patterning

The neural tube, once formed, undergoes a rough segmentation into brain and spinal cord in all vertebrates, through the formation of neuromeres, which are defined as distinctly swollen regions that are separated from each other by constrictions of the wall of the neural anlage (Kimmel, 1993). The regionalization of the brain into fore-, mid- and hindbrain components appears to occur in a similar fashion in zebrafish as other vertebrates. For example, the formation of the midbrain-hindbrain boundary is marked by the expression of an *engrailed* gene in zebrafish as in other vertebrates (Hatta *et al.*, 1991). Other transcription factors such as the products of *Krox-20* and *pax* genes are expressed in appropriate regions of the developing zebrafish brain as in other vertebrates (Kimmel, 1993).

Midline structures function in specifying neural fate

The cells of the embryonic shield of the zebrafish as well as the Spemann organizer of Xenopus not only function to produce signals required for patterning the early embryo, but also go on to form important structures at the embryonic midline (reviewed in Schier & Talbot, 1998). The notochord forms ventrally to the neural tube and is responsible for inducing the formation of the floor plate in the ventral midline of the neural tube (reviewed in Dodd, Jessell & Placzek, 1998).

The floor plate itself plays important roles in further patterning the nervous system through producing molecules required for the specification of neuronal fates (e.g. motoneurons & interneurons) and axon guidance (Dodd, Jessell & Placzek, 1998). The mechanisms responsible for specifying the floor plate seem to be fairly well conserved among vertebrates. Direct comparisons between genes involved in floor plate specification in zebrafish and those in other vertebrates are often obscured by the increased number of participating genes in the zebrafish. For example, *Shh* (sonic hedgehog) is required for the differentiation of the floor plate in the chick and mouse, however in zebrafish three *Shh* homologues, *sonic you, ehh* (echidna hedgehog) and *twhh* (tiggywinkle hedgehog) may be required to induce various regions of the floor plate (reviewed in Dodd, Jessell & Placzek, 1998).

Neuron specification

The earliest neurons in the zebrafish embryo arise in the center of each neuromere (Kimmel, 1993). It is important to note that the zebrafish possess two distinct types of neurons, primary and secondary. Vertebrates with larval forms, such as Xenopus, also develop primary neurons; mammals and birds that lack a larval stage of development do not. The primary neurons arise early in the embryo and establish a relatively simple nervous system used to generate behavior necessary for larval survival (Eisen, 1991;

Kimmel, 1993). Primary and secondary neurons differ in many ways. Primary neurons are larger and are individually identifiable through both their position in the soma and their stereotyped axon projections (Eisen, 1991; Kimmel, 1993). Studies have determined that the specification of both primary and secondary neurons (e.g. motoneurons) in the zebrafish is the same. Both primary and secondary motoneuron specification are dependent on signals from the midline, such as the products of the *Shh* gene homologues, as in other vertebrates (Beattie *et al.*, 1997). The difference between primary and secondary motoneuron specification concerns the presence of differentiated notochord or floor plate. In other vertebrates neuronal specification relies on the presence of these structures and the signals that they produce. Although these structures are not present in the early zebrafish embryo at the time of primary neuron specification, the same signals are produced by the progenitor cells of the notochord and activate the same downstream targets to effect motoneuron differentiation (Beattie *et al.*, 1997).

The main action of *Shh* gene products appears to be an alteration in the expression of various transcription factors in target cells (Eisen, 1998); different transcription factors ultimately regulate the expression of genes necessary to specify motoneuron subtypes. Both zebrafish and mouse motoneurons express the homeobox gene *islet1* at similar timepoints in development (reviewed in Beattie *et al.*, 1997). Cells that go on to produce cranial visceral motoneurons (Eisen, 1998) express *Nkx2.2* similarly in both zebrafish and other vertebrates (reviewed in Arendt & Nubler-Jung, 1999).

Axon guidance

As mentioned previously the floor plate functions as a source of signals not only to specify neuronal fates, but also to guide growing axons, for example by producing secreted netrins which can act as attractants or repellants (reviewed in Eisen, 1998 and Muller *et al.*, 1996). The zebrafish possesses two netrin genes, *net 1* and *net 2* (Strahle *et*

al., 1997) that are expressed by midline cells as the first commissural axons begin outgrowth towards the midline as in other vertebrate species (reviewed in Arendt & Nubler-Jung, 1999). Other guidance molecules such as the ephrins, which are membrane bound ligands for Eph receptors, appear to function similarly in zebrafish and other vertebrates to mediate repulsion of growth cones from incorrect paths via cell-cell contact (reviewed in O'Leary & Wilkinson, 1999). Guidance molecules can also function in part in establishing synaptic connections; for example, semaphorins can act as chemorepellants and can function to inhibit synapse formation (reviewed in Muller *et al.*, 1996). Semaphorins have been recently found in zebrafish and appear to function in a similar fashion (Shoji *et al.*, 1998).

Eye and retinal development

The development of the zebrafish eye begins differently than that of other vertebrates as a result of secondary neurulation. Solid optic lobes arise in the anterior region of the solid neural keel in the zebrafish in contrast to the optic vesicles observed in other vertebrate species (Malicki, 1999; Kimmel *et al.*, 1995). Regardless of this alternate beginning the remainder of eye morphogenesis in the developing zebrafish appears to be very similar to other vertebrates (Scmitt & Dowling, 1994).

At the molecular level the zebrafish appears to use the same signalling molecules as other vertebrates in establishing the structure of the eye and its various cell types (reviewed in Jean, Ewan & Gruss, 1998). For example, the early events in specification of the retina involve the *Pax-2* gene, which is thought to be activated by *Shh*. In both mice and zebrafish the lack of *Shh* leads to disruption in the development of the retina; in the zebrafish this ultimately leads to cyclopia. Defects in *Pax-2* (*noi* in the zebrafish) affect optic nerve development similarly in mice, zebrafish and possibly humans (reviewed in Jean, Ewan & Gruss, 1998).

Recent screens for developmentally important genes revealed many zebrafish mutants with defects resembling retinopathies even though retinal development and function is slightly different from that in humans (Malicki *et al.*, 1996).

Conclusion

It appears that the nervous system of the zebrafish develops similarly enough to that of other vertebrates to warrant its use as a model. The primary differences in its development concern gross morphogenetic movements while the underlying molecular mechanisms involved in nervous system development remain conserved. The "extra genes" of the zebrafish are another obvious difference, but do not pose a problem, rather the division of labor among multiple homologues may be useful in understanding the functions provided by single genes in mammalian systems. In the next section I will describe some specific advantages for the use of the zebrafish in the study of the development and function of the nervous system.

Advantages of the zebrafish as a model for vertebrate nervous system development

The zebrafish has many specific characteristics that set it apart from other vertebrate systems for investigating nervous system development and function. These features have been discussed in detail in many reviews (Kimmel, 1993; Fetcho & Liu, 1998; Fetcho & O'Malley, 1997; Schier, 1997; Dodd, Jessell & Placzek, 1998; Malicki, 1999; Schmitt & Dowling, 1999; Jean, Ewan & Gruss, 1999). I will summarize the highlights of these reviews below.

The nervous system of the zebrafish develops rapidly (described in Kimmel, 1993), and since development occurs external to the mother, events taking place in the optically clear embryo can be visualized in real time. The process of neural patterning begins around 9-10 hpf. A mere 14 hours later the morphogenesis of the brain is well advanced, the interconnections of the primary nervous system have been established and the embryo twitches involuntarily as initial synaptic connections begin operating. The nervous system of the zebrafish embryo is composed of primary neurons that have features that make them easily distinguishable from late arising adult (secondary) neurons (Eisen, 1991; Kimmel, 1993). The cell bodies of the primary neurons are very large (5-15 um) in comparison to the size of the embryo (50-100 um width at the trunk) (Kuwada & Bernhardt, 1990) and are easily observed in transparent embryos and larvae using a compound microscope with differential interference optics (DIC) (Eisen, 1991). The placement of the soma of the primary neurons and their axon projections are highly invariant from animal to animal (Westerfield & Eisen, 1988; Kuwada & Bernhardt, 1990; Eisen, 1991; Kimmel, 1993) making the study of unique, individual neurons *in vivo* possible. To date in other vertebrate systems (e.g. mouse and chick) only populations of neurons have been studied *in vitro* since identification of single neurons has not been possible (Eisen, 1991).

The primary nervous system of the zebrafish embryo is much simpler and contains fewer functional types of neurons than the complete adult nervous system (Eisen, 1991). The relative "simplicity" of the primary nervous system does not mean that it cannot generate complex behaviors; the zebrafish embryo and larvae participate in many behaviors such as the startle response, rapid swimming and escape from touch stimuli applied to the head or tail (Westerfield & Eisen, 1988; Fetcho & O'Malley, 1997). Techniques such as injection of fluorescent calcium dyes (to visualize neurons and monitor their activity) (Fetcho & O'Malley, 1997) and single cell laser ablations (Fetcho & Liu, 1998) are routinely performed in the zebrafish embryo. These techniques make it

possible to study how individual neurons contribute to behavior in a live animal and to identify which neurons are active during certain behaviors. A recent experiment, performed by Fetcho and Liu (1998), used laser ablation to kill a neuron previously hypothesized to play a role in a specific type of escape behavior. The ablation of this specific neuron led to the loss of tail touch elicited escape behavior, but not of head touch elicited behavior, confirming its role.

The ability to perform forward genetics in a vertebrate system allows us to find genes which have roles in nervous system development and function. The recent screens performed (Driever *et al.*, 1996; Haffter *et al.*, 1996), and ongoing screens at the University of Oregon and other institutions, have identified mutations involved in many aspects of neural development. The identified mutations range from those that affect early patterning in the nervous system to others affecting axon outgrowth and retinal development. In the mouse the standard approach has been to knock out genes with suspected roles in nervous system development or to knock out gene homologues with established roles in nervous system development in invertebrate systems. These studies, hampered with the inability to visualize primary defects in the developing nervous system, have not always been illuminating (e.g. subtle phenotypes, complex background effects).

The zebrafish is an excellent system in which studies of nervous system development and function can be conducted. The nervous system has been well characterized and is easily manipulated. The zebrafish is unique in that it is currently, the only vertebrate in which individual, identified neurons can be studied in a live, active organism.

The studies of the *unc-119* homologues of the zebrafish were initiated with the idea that the genes may have a wider role in the development and function of the nervous system than eluded to by the restricted expression in the retina of mammals (Higashide *et al.*, 1996). In the next section I describe the materials and methods used to explore the

expression pattern of the *unc-119* homologues in the embryonic and adult zebrafish. I then go on to discuss the results obtained by these methodologies, discuss their implications and propose future directions to be taken in this research.

Materials and Methods

Maintenance and care of zebrafish:

Maintenance and care of zebrafish was carried out as described in The Zebrafish Book (Westerfield, 1995) with modifications as outlined.

Adults:

For these studies adult zebrafish were defined as sexually mature fish (older than three months). Males are about half the size of females (under 1 gram in weight), and are slimmer, longer and darker in appearance. Females are larger (over 1 gram in weight), have whitish, full bellies and are slower moving. Information regarding age and fecundity of the fish was obtained through the supplier of the fish. Adult zebrafish (*Danio rerio*) were obtained at local pet stores in Edmonton, Alberta. Between 10–20 fish (equal numbers of males & females) were maintained in 40L tanks equipped with a thermostat (28-29°C), aerator and Aqua Clear mini filter system. A single clear 15 W aquarium bulb was used to provide 14 hours of light per 24 hour period. The use of this photoperiod ensures that a single clutch of embryos will be produced shortly after the onset of artificial dawn. When the correct photoperiod is not used embryos are produced at various times during the 24 hour period and thus develop asynchronously. The tanks were covered to prevent excess light from entering. On a weekly basis 10–20% of system water was removed from each tank and fresh water was added. System water was

prepared by adding 0.75 g/L aquarium salt (Nutrafin) to distilled water; 10 N NaOH was added to obtain a pH of approximately 7.0-7.5. Cycle (Rolf C. Hagen, Inc), a commercially available preparation of various bacterial species, was used weekly as suggested by the manufacturer to control ammonia and nitrite build-up. Tanks were scrubbed to remove alga and debris was siphoned out of tanks as required. Aqua Clear mini filters (ammonia, carbon and foam) were changed monthly in a staggered fashion. Ammonia and carbon filters were changed on the same day and the foam filter was changed alone two weeks later. Fish were fed Tetra Min Flake Food or frozen brine shrimp (prepared in system water) daily.

Embryos:

To collect embryos sterile marbles (bleached and rinsed extensively in distilled water) were placed in tanks the night prior to the day of collection. The layer of marbles provides a safe area where the embryos can develop without being consumed by adult fish. Adult fish will consume their own embryos under stressful conditions to recycle protein (e.g. the tank is an unnatural environment). Two hours after the light cycle commences on the day of collection, embryos are removed from the tanks by siphoning 4L of water from the marble covered bottom. After removing debris and discarding non-viable eggs, which appear opaque rather than transparent, the embryos are transferred to small (60 X 15 mm) petri plates at a density of 25 embryos per plate in embryo media (0.137 M NaCl, 5.4 mM KCl, 0.25 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 1.3 mM $CaCl_2$, 1.0 mM $MgSO_4$, 4.2 mM $NaHCO_3$). Embryos are maintained at 28.5°C ,which is the standardized temperature at which the development of embryos has been observed for construction of staging series, on a slide warmer (Kimmel *et al.*, 1995) . To suppress the development of pigmentation (pigmentation will obscure signals in *in situ* hybridization experiments) embryos were grown in embryo media with 0.2 mM PTU

(phenylthiocarbamide) beginning at 15 hpf. Embryos were staged according to hours or days post fertilization in addition to using a visual staging series (Kimmel *et al.*, 1995) since developmental rates vary even within a single clutch of eggs. Embryos were either flash frozen in liquid nitrogen and stored at -70°C (for RNA/DNA extractions) or fixed in 4% paraformaldehyde (made in 10X PBS-phosphate buffered saline) at 4°C overnight (for *in situ* hybridization experiments). For *in situ* hybridization experiments the embryos were transferred to 1X PBS and chorions were removed using #5 Dumont Tweezers (SPI Supplies) after fixation.

Larvae:

After 5 dpf the yolk sac is largely depleted and larvae must have food provided (Westerfield, 1995). To raise larvae a 10L tank was filled with system water and maintained at 25-29°C with a thermostat. The larvae do not require an aerator or filter system. Due to their small size waste build-up is not severe and the amount of dissolved oxygen in the water appears to be sufficient to support growth (see **Results** for further discussion). Larvae are not maintained on a photoperiod since they are not capable of reproduction. Larvae were fed Tetra Min Baby Fish Food, paramecium (*Paramecia multimicronucleatum*, Carolina Biological Supply) or frozen brine shrimp (prepared in system water). Paramecium cultures were grown in wheatgrass (Cerophyl Laboratories, Inc) (3g per 500mL of distilled water) at room temperature on the bench.

Degenerate oligonucleotide primed PCR to obtain zebrafish *unc-119* homologues:

A degenerate oligonucleotide primed PCR approach was used to identify *unc-119* homologues in species other than *C. elegans*. The degenerate primers MMA23 (C) and MMA25 (F) **(Table 2)**, were designed using conserved regions between HRG4 and UNC-119 proteins (Maduro *et al.*, 1999). 25 µl PCR reactions were prepared as follows:

1 µl of appropriate template was mixed with 1µl of each primer (100 pmol/µl), 2.5 µl of 10X PCR Buffer (USB), 1.5 µl of 25 mM $MgCl_2$, 4.0 µl of 1.25 mM dNTP mix, 13.5 µl of sterile H_2O and 1 µl of *Taq* polymerase (Amersham 5 U/µl).

Templates were either genomic DNA prepared from zebrafish embryos as described (Westerfield, 1995) or adult zebrafish cDNA prepared from whole adult male or female fish or unsexed heads (eyes removed) or unsexed bodies (eyes & heads removed) using total RNA and the 3' RACE kit (Gibco BRL) as suggested by the manufacturer.

A single 238 basepair product (*unc119a*) was obtained using genomic DNA as a template. This product was cloned into pBluescript II (KS-) (Stratagene) using BamHI sites introduced by PCR amplification (**Table 2**) and the insert was sequenced using Sequenase v.2.0 (Amersham) following the manufacturers' instructions (**Figure 4**). The resulting plasmid is referred to as pDP#*unc119a* (**Table 1**).

A 150 basepair product was obtained when all cDNA templates above (e.g. whole body, head without eyes, bodies without heads or eyes) were used in separate reactions. Products were cloned into pGEM-T (Promega) and six isolates were sequenced. Two classes of sequences (*unc119b* and *unc119c*) were obtained. The plasmids are referred to as pDP#W3unc-119 (corresponds to *unc119b*) and pDP#W5unc-119 (corresponds to *unc119c*) (**Table 1**).

Isolation of *unc119b* and *unc119c* cDNA clones:

A 15-19 hpf zebrafish embryonic cDNA library was obtained from Bruce Appel (University of Oregon, Eugene, OR, USA). The library was constructed using the lambda Uni-ZAP XR vector (Stratagene). cDNAs are cloned into the vector through EcoRI and XhoI sites. The library was maintained and screened as described in the manual provided by the manufacturer. Approximately half a million phage were

screened using inserts from pDP#W3unc-119 or pDP#W5unc-119 (**Table 1**). On average 10-25 positives were obtained in each primary screen. For each screen aliquots of positives from the first round were combined and diluted to perform a secondary screen. Pure isolates were then subjected to the *in vivo* excision protocol (Stratagene manual) to obtain phagemids carrying cDNA inserts in pBluescript. cDNAs were sequenced (**Figures 4, 5 & 6**) using the ABI PRISM system (Version 3.0) (Rick Rachubinski, University of Alberta) and T3, T7 or internal primers (BioServe, Biotechnologies, Laurel, MD, USA) designed as sequence was obtained (**Table 1**).

Northern analysis of zebrafish *unc-119* homologues

Total RNA was isolated from whole adult male and female zebrafish or bodyparts of unsexed adult zebrafish using RNAzol B (Teltest) according to the manufacturers' instructions and a Polytron electric tissue homogenizer (Brinkmann). Fish were quick frozen in liquid nitrogen and kept on dry ice until extractions were performed.

RNA gel electrophoresis and Northern blotting were performed essentially as described (Sambrook *et al.*, 1989). 1% or 1.6% formaldehyde agarose gels were prepared and loaded with ~ 5-25 μg of total RNA per lane (depending on the gel). RNA was transferred by capillary blotting in 10X SSC (SSC = 0.15 M NaCl/ 0.015 M Na_3•citrate pH 7.6) to GeneScreen Plus Membrane (NEN Life Science Products) and membranes were hybridized overnight in Hybrisol II (Oncor) solution. DNA probes were labelled with ^{32}P-dCTP using the T7 QuickPrime Kit (Pharmacia). Washes were performed at 65°C as follows: Twice 2X SSC 15 minutes, once 2X SSC/ 0.1% SDS (sodium dodecyl sulfate) 30 minutes, once with 0.1X SSC/ 0.1% SDS for 10 minutes.

***In situ* hybridization**

Whole mount *in situ* hybridization was carried out on zebrafish embryos of various stages as described with modifications (Detrich *et al.*, 1999a). Embryos were

fixed overnight in 4% paraformaldehyde (10X PBS) at 4°C. Embryos were washed in 1X PBS and dechorionated. Embryos were dehydrated through a series of methanol-PBT (phosphate buffered saline plus 0.2 % Tween-20) solutions (1:3, 1:1 and 3:1) and then washed twice in 100% methanol. Embryos were then stored at -20°C for at least 24 hours. Embryos were re-hydrated through a methanol-PBT series (3:1, 1:1 and 1:3) and then washed three times in PBT. Embryos over 24 hpf were digested with proteinase K (2 µg/mL in PBT) at room temperature for 5-45 minutes depending on the stage of development. All embryos were then re-fixed in 4% paraformaldehyde for 20 minutes at room temperature. Embryos were washed well in PBT and hybridized in standard hybridization buffer (60% formamide) at 70°C overnight with DIG-11-UTP (Boehringer-Mannheim) labeled probes (see below). Post-hybridization washes were carried out at 70°C as follows: Twice with 50% formamide, 1X SSC, 0.1% Tween-20 (referred to as solution "F") for 5 minutes, once with 2:1, 1:1 and 1:2 (F: 2X SSC/0.2% Tween-20) solutions for 5 minutes each, once with 2X SSC/0.2% Tween-20 for 5 minutes, twice with 0.2X SSC/0.2% Tween-20 for 30 minutes each. Embryos were equilibrated into PBT through a series of washes (3:1, 1:1 and 1:3 of 0.2X SSC/PBT) at room temperature. Embryos were blocked in 2 mg/mL BSA, 5% goat serum for at least 2 hours at room temperature. A 1: 10,000 dilution (0.075 U/mL) of digoxigenin-alkaline phosphatase Fab fragments (Boehringer-Mannheim) in blocking solution was added to embryos for incubation overnight at 4°C. Embryos were stained using 3.5 µl each of NBT (50 mg/mL, Promega) and BCIP (50 mg/mL, Promega) per mL of NTMT solution (0.1 M NaCl, 0.05 M $MgCl_2$, 0.1 M Tris pH 9.5, 0.1% Tween-20). When color had developed the reaction was stopped by washing with H_2O / 0.05% Tween-20. Embryos were fixed overnight at 4°C in 4% paraformaldehyde.

Probe synthesis:

DIG-11-UTP labeled RNA probes were prepared using the MegaScript Kit (Ambion) with slight modifications. Plasmids linearized with the appropriate restriction enzyme were used as templates. In some cases PCR was used to add a T7 RNA polymerase binding site to inserts and enzyme digestion was used to remove the original T7 site present. This strategy was used with pGEM-T (Promega) vectors when the insert was not available in both orientations. pGEM-T vectors have T7 and Sp6 RNA polymerase sites flanking the cloning site. The Sp6 RNA polymerase does not seem as robust, so to ensure approximately equal amounts of antisense and sense probes were obtained T7 sites were added when required. Plasmids pDP#W3unc-119, pDP#*unc119b*, pDP#*unc119c*, pDP#PSB5, and pDP#PDP2XhoI#1 were used as templates for the creation of sense and antisense probes. Control plasmids containing the full length Krox-20 cDNA and an uncharacterized cDNA expressed in somites (gifts of Yilin Yan, University of Oregon, Eugene, OR, USA) were prepared simultaneously and used to ensure hybridizations were successful and specific.

Linearized templates were incubated at 37°C for 2-6 hours with 0.5 µl RNase Inhibitor (Boehringer-Mannheim), 2 µl 10X nucleotide mix (10mM each of rATP, rCTP and rGTP, 6.5 mM of rUTP and 3.5 mM DIG-11-UTP), 2 µl 10X Transcription Buffer (Ambion), 2 µl T3 or T7 RNA polymerase (Ambion) and DEPC-treated water to a final volume of 20 µl. Reaction mixtures were then incubated with RNase free DNase at 37°C for 15 minutes. RNA probes were precipitated at -20°C overnight with 0.8 µl 0.5 M EDTA, 2.0 µl 5M LiCl and 75 µl 100% ethanol. Pellets were washed with 70% ethanol, air dried and resuspended in DEPC-treated water. Aliquots were run on agarose gels at high voltage to check quantity and quality.

Photography:

Embryos that have been stained can be stored indefinitely in 4% paraformaldehyde at 4°C. To prepare embryos for photography paraformaldehyde was removed and embryos were washed twice in 100% methanol and then transferred to 500 µl methyl salicylate. Methyl salicylate (Sigma, M-6752) functions to clear the embryo of background staining and thus, will diminish the signal over time so it is best to take the photograph as soon as possible after its addition. Embryos were transferred to a clear depression slide and excess liquid removed with a Kimwipe. Some photographs were taken with a Zeiss MC63 dissecting microscope equipped with a camera (M35W) and Schott KL 1500 light source to provide cold, fibre optic lighting from either side of the specimen. Other photographs were taken with a Zeiss Axioskop microscope equipped with a Spot camera and software. Composites of different focal planes were assembled in Adobe Photoshop version 5.0.

Results

Setting up the zebrafish

The experience of setting up a new organism in the lab was very frustrating, but ultimately rewarding. It required me to access many resources, be creative and to persevere when things were not going well. Although the details of setting up and maintaining the zebrafish have been published (Westerfield, 1995) each lab has different resources and constraints that require one to modify suggested approaches. In my own personal experience members of Dr. Zalik's lab (Department of Biological Sciences, University of Alberta), the zebrafish server (www.zfish.uoregon.edu), the zebrafish newsgroup (bionet.organisms.zebrafish), and later on in my project, members of the University of Oregon (Bill Trevarrow, Bonnie Ullmann and Charline Walker), were valuable resources for information on fish breeding and raising of embryos.

Since adult fish are quite hardy and appear healthy (e.g. actively swimming and schooling, upright fins rather than drooping fins, etc.) under a range of conditions, it isn't always apparent why they are not producing embryos or embryos are developing asynchronously or not surviving well. I will discuss some of the parameters that should be checked when encountering difficulties.

The most important factor in raising the zebrafish and keeping them in good breeding condition is water quality. Unfortunately, a water supply is never completely constant even when obtaining filtered, pure water (e.g. dH_2O). Seasonal fluctuations of unknown factors (e.g. chemical or biological), which may not be removed by filtration devices, can affect the fecundity of the fish. During the past three years I have noticed that during spring run-off embryo production and survival of embryos drops. Normally a tank of 12 - 25 fish will produce up to 1000 embryos with survival up to 80 %; during spring run-off this can drop as low as 25 - 100 embryos per collection. In many cases

these embryos would not survive beyond the first day. To circumvent these seasonal fluctuations I have stored dH_2O from other times of the year to use during spring run-off.

Another important factor for fish health is keeping the pH of the tank between 7.0 and 8.0 and maintaining control of the ammonia and nitrite waste products that build up in the tank. If the pH of the tank water is too low fish will absolutely not breed although the adults may appear healthy. System water should be prepared exactly as described in **Materials and Methods**. Alternate sources of water may have a lower starting pH (e.g. Milli-Q) or different filtration systems may result in the removal or addition of factors that are unidentified, but necessary for fish health. Even obtaining dH_2O from another floor of the same building can be a problem. The pipes that the water has traveled through are different from floor to floor.

Other commercially available salts (e.g. Instant Ocean) are recommended by many zebrafish resources, however I have found that Nutrafin aquarium salt is sufficient. Different additives in other salt preparations may affect the fish adversely or react poorly with other components of the system (e.g. bacterial species responsible for maintaining healthy nitrite or ammonia levels). With nitrite and ammonia problems often massive and sudden fish death will occur (e.g. if filters are changed too frequently and bacteria levels drop too quickly) or a low level of death over a period of time will be observed (e.g. one fish a day for a week dies). Under these circumstances a sample of system water can be taken to a pet store and tested for ammonia and nitrite levels. An extra dose of Cycle, which contains various bacterial species that control ammonia and nitrite levels, (**Materials and Methods**) may help bring toxic levels back to the normal range quickly.

Asynchronous development of zebrafish embryos (e.g. upon collection you immediately see embryos ranging in age from 2 hpf up to 48 hpf) suggests that the light cycle is not correctly set up. If the light cycle is not providing 14 hours of light and 10 hours of darkness adults will spawn at any time during the light cycle resulting in embryos that are developing asynchronously. If the tank is not light tight and light is

entering during "lights off" unregulated spawning will also occur. If a transparent bulb is not used (e.g. colored bulbs should not be used) the fish will spawn at any time during the light cycle even if the correct photoperiod is being provided. The fish seem to be sensitive to the intensity and wavelength of the light in addition to the cycle of light and dark (Charline Walker, pers. comm.).

Embryos of "poor quality" will sometimes be observed. For example, high numbers of embryos may be obtained that appear healthy initially, but tend to degenerate within the first day. Always make a mental note of the appearance of non-viable eggs for each collection. Non-viable eggs from healthy females will have a white, opaque appearance. Non-viable eggs from females that are nearing the end of their reproductive life will be yellowish and opaque. It appears that such older females may reduce the fecundity of their younger tank mates, perhaps through chemical signals (Bill Trevarrow, pers. comm.). When this situation is encountered it is necessary to identify the female(s) responsible and remove them from the colony. Older females will appear larger and their bellies will be noticeably pronounced.

Adding new, young fish (e.g. up to 25 per 40 L tank total is the upper limit) can increase embryo production. New fish should not be added directly to the tank, they may carry diseases that the colony has not previously encountered or do not have resistance against. Rather, new fish should be kept in a holding tank overnight and observed. To further ensure that new fish are suitable to introduce to the current colony they should be treated them in an atabrine solution overnight; healthy fish will survive this treatment (Westerfield, 1995; Charline Walker, pers. comm.). It is important that other "fish tonics" are not used since atabrine is the only chemical that has been found to be non-mutagenic (Charline Walker, pers. comm.). Seperate tanks in the same facility should also be kept isolated to avoid the spread of disease. Siphons, nets and other tank accessories should be used for one tank only.

Raising fish to sexual maturity (beyond three months) has not been a successful venture to date. Only a handful of fish have been raised beyond eight weeks. Many unidentified factors appear to be involved in these experiments. Highly controlled experiments designed to raise zebrafish embryos in space through N.A.S.A have not been successful (Barbara Chapman, University of California, Davis, bionet.organisms.zebrafish posting, August, 1999). Additional postings to the zebrafish newsgroup from various researchers worldwide confirm that beyond three weeks post fertilization zebrafish larvae do not survive well in general and in some cases seem to actually experience a massive death at this time point. We have tried various strategies to resolve this problem (e.g. decrease density of larva, different food, various water levels, etc.) and continue to search for ways to solve it.

There are at least three *unc-119* homologues present in the zebrafish:

Degenerate primers designed against the most highly conserved regions in the carboxyl termini of HRG4 and UNC-119 (Maduro *et al.*, 1999) amplify three *unc-119* homologues in the zebrafish. pDP#*unc119a* (**Table 1**) contains the 238 basepair product obtained when the degenerate primer set , MMA23 and MMA25 (**Table 2**), is used on zebrafish genomic DNA. *unc119a* contains a putative 85 nucleotide intron based on the occurrence of the consensus splice donor and acceptor sequences GT/AG at the same position as in other *unc-119* gene homologues (Morris Maduro, pers. comm.) and the presence of in-frame stop codons in the intron (**Figure 4**). pDP#W3unc-119 and pDP#W5unc-119 contain the two unique 150 basepair products obtained when MMA23 and MMA25 primers are used on zebrafish adult cDNAs made from general dissections of body parts from both sexes (e.g body without head or eyes and head without eyes). The plasmid pDP#W3unc-119 contains a 150 basepair sequence that corresponds to the *unc119b* gene and the plasmid pDP#W5unc-119 contains a 150 basepair product that

corresponds to the *unc119c* gene (see below). Genomic sequences corresponding to the partial sequences of *unc119b* and *unc119c* were not found in the PCR reaction using zebrafish genomic DNA as a template; this is not surprising since only one clone arising from the transformation reaction was analyzed. Other genomic products could easily have gone undetected. Alternatively, *unc119b* and *unc119c* could contain larger introns at the same position as the intron of *unc119a* and therefore, would not be favored amplification products in the reaction because of their greater size. Also, either MMA23 or MMA25 may be unable to bind to *unc119b* and *unc119c* sequences if introns are present that disrupt the primer sequences. It is certain that sequences corresponding to *unc119b* and *unc119c* are present in the genome and have simply gone undetected in this work. It is not surprising that there are multiple *unc-119* homologues in the zebrafish considering that its genome underwent a partial or complete duplication after its divergence from the lineage leading to mammals (Amores *et al.*, 1998; Postlethwait *et al.*, 1998; reviewed in Meyer & Malaga-Trillo, 1999). As previously noted an additional human *unc-119* homologous sequence has been deposited in the EST database (Dave Pilgrim, pers. comm., 1999); this suggests that the possession of multiple *unc-119* homologous genes is common in vertebrates (**Figure 2**).

A 15-19 hpf embryonic zebrafish cDNA library was screened to obtain larger sequences corresponding to *unc119b* and *unc119c*. cDNAs corresponding to each of the partial PCR products were found (**Figure 4, 5 & 6**). pDP#*unc119b* (**Table 1**) is a plasmid carrying the 1.3 kilobase cDNA that contains the sequence of the PCR product of pDP#W3unc-119. pDP#*unc119c* (**Table 1**) is a plasmid carrying the 1.7 kilobase cDNA that contains the sequence of the PCR product of pDP#W5unc-119. Open reading frames (ORFs) were assigned to the zebrafish *unc119b* and *unc119c* cDNA sequences based on homology to other UNC-119 family members (**Figure 2**), however no in-frame stop codons are present upstream of the first methionine codon chosen. Therefore, it is not absolutely certain that the *unc119b* and *unc119c* ORFs are complete. If the chosen ORFs

do represent the complete coding sequence of the *unc119b* and *unc119c* genes the UNC119B protein would be the smallest of the family at 206 amino acids and UNC119C would be 3 amino acids longer (**Figure 2**) than HRG4 and RRG4 (240 amino acids).

***unc119a* does not appear to be expressed in adult zebrafish:**

Sequences corresponding to *unc119a* have not been detected when the primers MMA23 and MMA25 are used on adult cDNA templates. 5' RACE using primers specific to *unc119a* and adult total RNA did not yield a product. These results suggest that *unc119a* may not be expressed in the adult zebrafish. It is still a possibility that *unc119a* is expressed in embryonic zebrafish. Alternatively, it is possible that *unc119a* is a pseudogene and is not expressed at all. Further work must be done to resolve these questions. Screening of the available embryonic cDNA library with *unc119a* and repeating 5' or 3' RACE using various embryonic cDNAs as templates and primers specific to *unc119a* will be performed.

***unc119b* and *unc119c* are expressed in adult male and female zebrafish:**

Northern blots prepared from 1.6 % gels containing ~25 ug per lane of total RNA isolated from whole bodies of female (F) or male (M) zebrafish were used to analyze the expression of *unc119b* and *unc119c*. Although RRG4 was not found to vary in its expression level over the 24 hour period (Higashide *et al.*, 1996) we wished to address whether any of the zebrafish *unc-119* homologues were expressed in a differential manner over the 24 hour period. To detect circadian expression RNA was prepared from three time points of adult sacrifice. Sacrifices were carried out at 9:30 am (A), 5:30 pm (B), and 9:30 pm (C) (**Figure 7A**). Alternatively, on an independent Northern a mixture of RNA prepared from all time points was used (M or F) (**Figure 7B**).

Hybridization with the complete insert of pDP#*unc119b* (**Table 1**) results in the detection of at least two transcripts in adult zebrafish (**Figure 7A & B**). One transcript (2.4 kilobases) is more highly expressed in females as supported by the actin loading control which clearly demonstrates the greater quantity of male RNA present (**Figure 7B**). The 2.4 kilobase transcript present in males and females does not appear to fluctuate greatly in expression level over the 24 hour period as compared to the amount of 18S ribosomal RNA present (**Figure 7A**). However, densitometric measurements are needed to accurately confirm any statements concerning this blot. The smaller transcript at 1.7 kilobases is barely, if at all, visible in the male lanes of either blot. This suggests that in the adult zebrafish the 1.7 kilobase transcript could represent a female specific transcript that does not appear to fluctuate in expression over the 24 hour period (**Figure 7A**).

Hybridization with the complete insert of pDP#*unc119c* (**Table 1**) results in the detection of at least three transcripts in adult zebrafish. The transcript sizes are 5.4, 3.3 and 1.9 kilobases approximately (**Figure 8A & B**). The smallest transcript at 1.9 kilobases is not visible in any male lanes. The 1.9 kilobase transcript is present in all female lanes and does not appear to fluctuate in expression level over the 24 hour period as compared to the amount of 18S ribosomal RNA present (**Figure 8A**). Again, densitometric measurements would be required in order to fully validate this observation. The 3.3 kilobase transcript is present in all female lanes with no apparent changes in expression level over the 24 hour period (**Figure 8A**). The 3.3 kilobase transcript is only faintly visible in males although the actin loading control demonstrates a greater quantity of male RNA than female RNA (**Figure 8B**). In the female the 5.4 kilobase band is most apparent in the "FC" lane which corresponds to the 9:30 pm sacrifice, however in the male the 5.4 kilobase band is most apparent in the "MA" lane which corresponds to the 9:30 am sacrifice. It must be kept in mind that the female 5.4 kilobase transcript is not necessarily identical in content to the male 5.4 kilobase transcript. They may simply

share the region of sequence homology that cross-reacts with *unc119c* and be very different otherwise. This observation suggests that the 5.4 kilobase transcript(s) may have a circadian function that differs in the two sexes.

Neither *unc119b* or *unc119c* cDNA sequences contain an in-frame stop codon upstream of the sites I have designated as ORFs based on alignment with homologues and the position of the first methionine codon. The smallest transcript detected through adult Northern analysis with an *unc119b* probe is 1.7 kilobases (**Figure 7A & B**); the embryonic cDNA is 1.3 kilobases therefore, it appears that it may not be full-length. The smallest transcript detected through adult Northern analysis with an *unc119c* probe is 1.9 kilobases (**Figure 8A & B**); the embryonic cDNA is 1.7 kilobases also suggesting that the full-length cDNA has not been captured. Alternatively, the embryonic splice forms may indeed be smaller than the adult splice forms. 5' RACE reactions will resolve these possibilities.

Overall, it seems unlikely that the higher level of expression of *unc119b* and *unc119c* observed in the female is artifactual since four independent Northern blots have produced the same result and actin probing on two of the blots show that male RNA is intact and present in a higher quantity than in the female lanes (**Figure 7B & 8B**). In addition a female specific gene identified in our lab, PDP2 (pyruvate dehydrogenase phosphatase) (Paul Stothard, upub. results) demonstrates what a completely female specific pattern looks like (**Figure 9**) in contrast to the variations in expression pattern of some of the *unc119b* and *unc119c* transcripts. It is not known why females would have a higher level of expression of *unc119b* or *unc119c* genes since they are not predicted to be sex-specific based on observations regarding *unc-119* homologue expression in other systems.

Regardless of the puzzling differential sex expression how can one account for all of the transcripts detected? They could be splicing variants or cross-reacting transcripts of additional *unc-119* homologues in the zebrafish genome. *unc119b* and *unc119c* do not

strongly cross-react with each other under high stringency conditions (65° C) based on the observation that Northerns do not show common transcripts and the sequences did not cross-hybridize during library screening. This is not surprising considering that even over the smallest region (e.g. 150 nucleotides) that was used to create probes they are only 70% identical (**Figure 4**). Another possibility could explain the abundance of multiple transcripts. Many pseudogenes could have been created after the genome duplication and subsequent evolution. Perhaps the most strongly hybridizing bands represent transcripts from functional genes and the weaker hybridizing bands represent transcripts from non-functional genes. It would be useful to look at the developmental expression of *unc119b* and *unc119c*, particularly to determine if the multiple transcripts observed in adult stages are detected at specific embryonic stages. Northern analysis using RNA isolated from various embryonic stages could be used to address this possibility.

***unc119b* and *unc119c* are expressed in embryonic zebrafish:**

The detection of the two zebrafish *unc119* cDNAs in an embryonic library prepared from 15-19 hpf zebrafish embryos suggests that *unc119b* and *unc119c* may be involved in a more general role than one in photoreceptor cell function in the eye. The retina of the zebrafish does not begin forming until after 24 hpf, photoreceptor cells are not generated until approximately 40 hpf, and classes of photoreceptor cells are not morphologically distinct from one another until 4 dpf (Malicki, 1999). It is possible that additional as yet unidentified zebrafish *unc-119* homologue(s) are expressed solely in the eye.

Embryos ranging from 2 hpf – 100 hpf were used to perform whole mount *in situ* hybridizations. Krox-20 and somite controls were always performed in conjunction with each experimental trial and were always successful (**Figure 10**). Krox-20 is expressed in

rhombomeres 3 and 5 of the developing hindbrain from approximately 11 hpf to 24 hpf; the somite specific transcript is expressed in somites from 12 hpf to 48 hpf (Yilin Yan, pers. comm.). In addition, *in situ* hybridization with PDP2 demonstrates a general staining pattern in embryos and also serves as a control for the specificity of the *unc119b* and *unc119c* reactions (**Figure 12 A-E**). Although PDP2 is female specific in adults it does not appear to be sex specific in embryonic stages.

The use of the 150 basepair insert from pDP#W3unc-119 or 1.3 kilobase insert from pDP#*unc-119b* (**Table 1**) as probes for these experiments resulted in detection of a transcript from 6 hpf up to 75 hpf (**Figure 11 C, D & F**). The use of the 1.7 kilobase insert from pDP#*unc-119c* (**Table 1**) as a probe for these experiments resulted in detection of its expression from 6 hpf to 34 hpf (**Figure 11 A & B**). Since the photography of embryos was difficult stages that resulted in the clearest pictures were used. In *unc119b* trials both antisense and sense probes in older stages (e.g. 96 or 100 hpf) did not stain embryos suggesting that the probe is not penetrating the tissues otherwise a background level of staining would be evident in the sense control. For stages older than 34 hpf sense and antisense probes from *unc119c* were also variably unable to produce staining (**Figure 11 E**) also suggesting a problem with probe penetration. Given the expression of the *unc119b* and *unc119c* transcripts in adults it is likely that the lack of staining is due to incomplete probe penetration. Various proteinase K digestion times could be tested to determine if this is indeed the problem. The PDP2 trials show a marked difference between sense and antisense staining even at much older stages (e.g. 61 hpf) (**Figure 11 E**), however a different batch of proteinase K was used for the *in situ* experiments for these embryos. An alternative explanation is that the variability in *unc119b* and *unc119c* staining could be a result of sex differences between embryos; males may not express high enough levels of these transcripts to be detected (see above Northern analysis section for discussion). Sex does not become determined in zebrafish embryos until 24 hpf (John Postlethwait, pers. comm.) so this possibility could

be tested by looking for equal numbers of *unc119b* or *unc119c* staining and non-staining embryos beyond this stage.

The pattern of expression for *unc119b* and *unc119c* are indistinguishable at this level of analysis; photographs of stained embryos are representative for each other. The most prominent staining is observed in the brain and developing fin bud for both *unc119b* and *unc119c* and appears to be occurring in nerve processes (**Figure 11 C & D**). If the staining reaction is allowed to proceed for a longer period of time (e.g. overnight) it appears that the transcript is also present at lower amounts throughout the body presumably in neuronal tissue (**Figure 11 A & B**). The lower level expression has a diffuse appearance, therefore cross-sections are required to resolve the localization of these transcripts more clearly. Unfortunately, the preparation of cross sections can often result in the loss of significant amounts of signal. To circumvent this problem fluorescein labeled probes may be used in conjunction with optical cross-sectioning by confocal mircroscopy (Detrich *et al.*, 1999a).

Although there is evident staining of the brain and developing fin bud in the zebrafish embryo, this staining does not appear to extend to the eye, although low level staining may go undetected (**Figure 11 C & D**). Examination of PDP2 staining in 61 hpf embryos demonstrates prominent staining of the eye as compared to the sense control (**Figure 12 E**) which would support the conclusion that staining of the eye is not occurring with *unc119b* or *unc119c* reactions where sense and antisense probes result in the roughly the same amount of signal in the eye (**Figure 11 F**). Cross sections prepared from *unc119b* and *unc119c* embryos observed at high power should be examined to resolve whether expression is occurring in the eye and is simply undetectable at the current level of observation (e.g. possible restriction to photoreceptor cells as with HRG4/RRG4).

Discussion

The *unc119a, unc119b,* and *unc119c* genes of the zebrafish are homologues of the *unc-119* gene family at the sequence level

In an alignment of the conceptual translation of *unc119b* and *unc119c* sequences with other UNC-119 family members (**Figure 2**) it is obvious that the amino acid sequences are more highly conserved in the carboxy termini of the proteins than the amino termini. It is interesting that UNC119B shares the motif "MKVKKG", at the beginning of its putative coding region, with HRG4 and RRG4. The amino termini of this family of proteins are very poorly conserved overall, yet the human, rat and zebrafish (UNC119B) proteins share this "motif" with 420 million years of evolution separating fish and mammals (Postlethwait & Talbot, 1997). This motif may be important for a specific function in vertebrates since the deletion of this motif does not affect the ability of HRG4 to rescue *unc-119* mutant worms (Maduro *et al.*, 1999). Perhaps the motif has a subtle role in regulating the activity of the protein.

Are there additional zebrafish *unc-119* homologues?

The observation that humans contain two *unc-119* homologues, HRG4 and a newly identified human EST (Dave Pilgrim, pers. comm., 1999) and that a genome duplication occurred in the zebrafish suggest that it is possible that zebrafish could contain additional *unc-119* homologues. Two approaches could be used to search for such additional sequences, PCR using degenerate oligonucleotide primers designed against highly conserved amino acid regions could be used since as mentioned in the **"Results"** section only one PCR product was analyzed in the original genomic PCR experiment. Alternatively, the *unc119b* and *unc119c* cDNAs could be used under low stringency conditions as probes on the available cDNA library to identify cross-hybridizing sequences. To obtain corresponding genomic sequences a zebrafish genomic

library could then be screened with these cDNAs or PCR using specific primers and genomic DNA could be performed. It would be useful to identify promoter regions and control elements of these genes to look for clues as to their functions as well as for the creation of expression constructs to further investigate the pattern of expression.

Are *unc119b* or *unc119c* functional homologues of *unc-119*?

HRG4 has been shown to be capable of completely replacing *unc-119* function in *unc-119* mutant worms (Maduro *et al.*, 1999). Zebrafish *unc119b* and *unc119c* will each be tested for their ability to rescue the multiple defects of *unc-119* mutant worms. The sequences corresponding to the putative open reading frames of *unc119b* and *unc119c* are currently being cloned into the same expression vector used for HRG4 rescue experiments (Maduro *et al.*, 1999). Based on the strong conservation of the carboxy termini of these proteins and the demonstrated lack of necessity for many amino terminal amino acids of HRG4 for *unc-119* rescue (Maduro *et al.*, 1999) it is expected that the zebrafish *unc-119* homologues will be capable of rescue. If they are not capable of rescuing *unc-119* mutants it may indicate particular amino acids in the carboxy terminus that are absolutely required for proper functioning of the protein. This may help to further delineate important "domains" in these proteins.

Is *unc119a* expressed in the embryonic or adult zebrafish?

unc119a was the first zebrafish *unc-119* homologue discovered, but its expression pattern has not been determined. Initial experiments did not meet with success and since progress was rapid with the other homologues, *unc119b* and *unc119c*, they were studied more extensively. 5' RACE with *unc119a* specific primers has been attempted using adult cDNA as the template, but was unsuccessful, however the appropriate controls were not performed therefore, these results are inconclusive. The use of the partial *unc119a*

sequence as a probe on adult Northern blots was not successful, however the use of the 150 basepair *unc119b* and *unc119c* fragments did not produce signals either, only full length cDNA probes produced signals. *unc-119a* may be expressed in embryos, however this possibility has not been explored. To address this question the available cDNA library could be screened with the available *unc119a* sequence. In addition the 5' RACE experiment should be repeated using adult cDNA and various embryonic cDNAs as a template to explore the expression of this homologue.

Are *unc119b* and *unc119c* cDNAs full length?

Since neither *unc119b* nor *unc119c* cDNAs have in frame stop codons in their upstream sequences it is uncertain whether the cDNAs contain full length open reading frames. In addition, the sizes of the smallest transcripts, detected through adult Northern analysis (1.7 & 1.9 kilobases for *unc119b* and *unc119c*,respectively), indicate that the *unc119b* and *unc119c* embryonic cDNAs may not be full length. The embryonic transcripts could be genuinely smaller than the adult transcripts or the adult transcript sizes could represent full length. 5' RACE has been attempted, but has not yielded clear results; the primary difficulty has been in determining an appropriate annealing temperature for the primers. These experiments will be repeated.

Developmental Northern and Western analysis

Given the number of transcripts detected by *unc119b* and *unc119c* sequences it would be useful to investigate if certain transcripts are present only at particular times in development through performing developmental Northern analysis. Perhaps certain transcripts are expressed only in embryonic stages and others in adult stages. The production of antibodies would allow the proteins to be analyzed in a similar fashion.

Apparent sex differential expression of the zebrafish *unc-119* homologues?

Based on observations in *C. elegans*, rat, and human, the *unc-119* gene and its homologues are not predicted to have sex specific expression patterns. The adult Northern analysis indicates that the *unc-119* homologues are more highly expressed in adult female zebrafish; some transcripts are not even detectable in the male (e.g. the 1.9 kilobase *unc119c* transcripts). It is possible that the zebrafish genes *unc119b* and *unc119c* have sex specific functions and that other, as yet undiscovered, homologues perform identical non sex-related functions in both sexes. Many researchers have studied the establishment of sexual dimorphism in the nervous system. Primarily, the action of steroid hormones can alter many mechanisms in nervous system development such as neurogenesis and synapse formation (Cooke *et al.*, 1998). Perhaps *unc119b* and *unc119c* are downstream targets of such hormones.

Temporal expression of the *unc-119* homologues and events in development

The earliest detected expression of *unc119b* and *unc119c* is in embryos 6 hpf. The midblastula transition, the time point when zygotic transcription begins, occurs at approximately 3 hpf (Kimmel *et al.*, 1995) therefore the zebrafish *unc-119* homologues do not appear to be maternally provided. The first detected expression of *unc119b* and *unc119c* occurs well before overt patterning of the nervous system which begins at approximately 9 hpf (Kimmel, 1993). The staining reaction of the *in situ* hybridizations were allowed to proceed for varying lengths of time depending on the particular trial. The most prominent staining is always first observed in the brain and fin bud (**Figure 11 C & D**). When staining reactions are allowed to proceed for much greater lengths of time both *unc119b* and *unc119c* appear to be expressed throughout the remainder of the body at a lower level (**Figure 11 A, B & F**). It is impossible to localize the expression to

neuronal cells specifically; sections of whole mount embryos would have to be prepared to determine the precise site of expression.

Raising antibodies to zebrafish UNC119 proteins

To begin exploring the function of the UNC119 proteins in the zebrafish it will be necessary to raise antibodies to these proteins. It may be useful to raise antibodies to each protein by expressing their disparate amino termini therefore the resultant expression pattern should represent each proteins' unique localization pattern and not an overlapping pattern of the two (or more) proteins involved. In conjunction, the RRG4 antibody has been received and will be tested on a Western blot containing zebrafish protein extracts to determine its usefulness. To ascertain the localization pattern of UNC119B and UNC119C proteins it may be best to perform antibody staining on whole-mount embryos and then section the animals as is commonly done (see chapter 6 in Detrich *et al.*, 1999b).

Mapping the zebrafish *unc-119* homologues

It will be of interest to map the zebrafish *unc-119* homologues. Another zebrafish gene, PDP2, has been successfully mapped (Paul Stothard, unpublished results) using a radiation hybrid panel provided by Marc Ekker and colleagues (Hukriede *et al.*, 1999). Since the zebrafish and human genomes share extensive regions of synteny (Postlethwait *et al.*, 1998) it is possible that some of the zebrafish *unc119* genes will lie in a region corresponding to 17q11, the position of the HRG4 gene (Higashide & Inana, 1999; Swanson *et al.*, 1998). If so this would facilitate studies of the evolution of this gene family, particularly aspects of gene duplication and retention or sub-functionalization of gene duplicates (Force *et al.*, 1999). Perhaps one homologue has retained the ancestral function similar to that in invertebrates (e.g. functioning in the nervous system as a

whole) and other homologues have gained more specific functions (e.g. photoreceptor cell function).

Production of GFP fusion constructs:

An extremely useful tool in studies of the development and function of the nervous system would be a GFP (Green Fluorescent Protein) reporter expressed throughout the nervous system of the developing zebrafish embryo. To examine the expression pattern of *unc119b* and *unc119c* in the nervous system a GFP fusion construct could be used to create transgenic zebrafish. To accomplish this a genomic library would be screened for *unc119b* and *unc119c* in order to obtain their promoter sequences and the appropriate constructs would be injected into early stage embryos. Alternatively, modified PCR reactions (e.g. inverse PCR) could be used to isolate upstream regions of the genes. GFP constructs would be useful for visualizing the expression of these genes and if one or both are expressed throughout the nervous system such a reagent would be desirable to those currently using injection of fluorescent calcium dyes to study neuron function in the zebrafish (Fetcho & Liu, 1998). Transgenic zebrafish have been constructed in which GFP is expressed in other cell types, such as blood cells (Long *et al.*, 1997), but to date a neural specific marker has not been developed (Fetcho & Liu, 1998).

Using antisense technology to establish roles for the *unc-119* homologues in zebrafish

In *C. elegans* the technique of RNA-I (RNA Interference) has been used to generate mutants by disrupting gene function through injection of double-stranded RNA corresponding to the target desired (Fire *et al.*, 1998). Similar techniques to reduce the amount of a target mRNA have been utilized in the zebrafish with success (see chapter 7

in Detrich *et al.*, 1999a). For example, the *alx* gene of zebrafish has been inactivated by injection of antisense oligonucleotides and results in a phenotype similar to mice with ocular retardation caused by a mutation in the murine *alx* homologue (Barabino *et al.*, 1997). It would be relatively easy to perform such an experiment using portions of or complete *unc119b* and *unc119c* cDNAs. The phenotype resulting from such experiments may help determine the role of these genes and how their actions differ from each other or if they are redundant.

Concluding Remarks

Based on the data from studies of *unc-119* in *C. elegans* and mammalian homologues one could suggest that the members of this family of genes function in the zebrafish in axon outgrowth during development as well as synpatic vesicle recycling. In zebrafish the early function of these genes could be that of axon outgrowth with a later restriction to photoreceptor cells of the eye of the adult where its function would involve synaptic activity. This type of restriction in expression throughout development has been observed for other zebrafish genes. The *vsx1* gene of the zebrafish is expressed first in the developing central nervous system (e.g. neurons of the hindbrain and spinal cord). During the course of development *vsx1* ultimately becomes restricted to a single cell layer of the retina (Passini *et al.*, 1998). Alternatively, the zebrafish could possess multiple *unc119* genes some with functions in axon outgrowth and others with functions specific to the eye.

Since expression of the zebrafish *unc119b* and *unc119c* genes is detected many hours prior to the initial patterning of the nervous system (e.g. 9 hpf) or axon outgrowth (18 hpf) it is not certain what role these genes may be playing at this very early stage. Detection of a transcript does not necessarily reflect the presence of the corresponding

protein. In *C. elegans unc-119* is also expressed very early in the embryo in presumptive neurons prior to axon outgrowth for as yet unknown reasons (Maduro *et al.*, 1995).

The observation that the most prominent expression occurs in the brain and developing fin bud of the zebrafish embryo suggests that *unc119b* and *unc119c* could function in the establishment of the primary nervous system of the embryo. The primary nervous system functions to provide the larva with basic survival behavior and the fins of the animal play a major role in movement, therefore the prominent staining in the fin bud would not be surprising in this proposed scenario (Sara Zalik, pers. comm.). The early innervation of the fin bud would be required to coordinate escape behavior. The above suggestion does not exclude the requirement for the *unc119* genes in the adult zebrafish. The function of the *unc119* genes may be general to all embryos in the establishment of the primary nervous system. However, later in development as sex determination occurs the *unc119* genes may take on more specialized roles (e.g. alternately spliced, sex specific transcripts) and function to establish sexual dimorphism in the nervous system as the permanent adult (secondary) nervous system is formed. The sex determination pathway may result in the production of steroid hormones that activate downstream targets required in creating the sexually dimorphic nervous system (Cooke *et al.*, 1998).

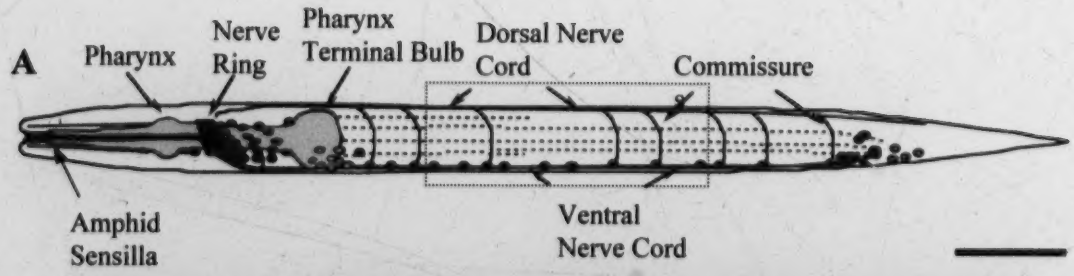
In the sexually mature adult (e.g. 3 months or older) differential expression of *unc119* genes throughout the 24 hour period could reflect two possiblities. If there are *unc119* genes with eye specific expression their protein products may function to transmit information regarding the photoperiod to downstream targets. Alternatively, the *unc119* genes may be upregulated by other factors involved in sensing changes in the photoperiod and the *unc119* gene products themselves may function to create an appropriate output. These genes may be involved in a pathway that links changes in the light cycle to reproduction by acting through the sexually dimorphic nervous system. Such links have been studied in mammals where the rhythm of melatonin secretion by the pineal gland (an evolutionary relative of the eye) appears to be under the control of

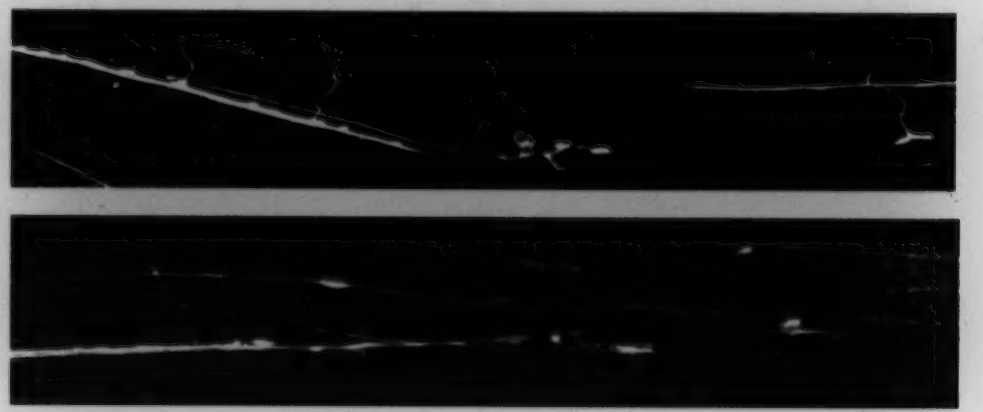
circadian oscillators (responsive to light and other environmental signals) (Goldman, 1999). The rhythm of melatonin production may function in regulating behavior and physiological changes (Goldman, 1999). Circadian rhythms are just beginning to be analyzed in the zebrafish at the molecular level (e.g. Gothilf *et al.*, 1999); further work must be done to address whether the zebrafish *unc119* genes could be functioning in circadian pathways.

Regardless of the speculations that have been spawned by the initial characterization of the *unc-119* homologues in the zebrafish the immediate goal of this thesis has been met. The main goal was to examine the expression pattern of the zebrafish homologues of the *C. elegans unc-119* gene in order to address the apparent discrepancy in the expression pattern of the mammalian homologues. The analyses of mammalian homologues initially focused on aspects of retinal expression, however, recent data supports the expression of HRG4 in non-retinal and embryonic tissues (Swanson *et al.*, 1998) which in further supports the data collected in this thesis. The initial characterization of two of the zebrafish *unc-119* homologues has been successful in establishing expression of this family of genes in non-retinal tissues during embryonic development and in adults.

The continued studies of the *unc119* genes of the zebrafish should aid us in reaching our ultimate goal in understanding the development and function of the nervous system. Directly, these studies will add to our understanding of the basic mechanisms underlying nervous system development in vertebrates and indirectly, these studies may provide valuable reagents for the correlation of neuron function and behavior (GFP constructs) as well as insights into other aspects of vertebrate biology such as circadian rhythms and sexual dimorphism.

Figure 1 ***unc-119*** **mutants have disorganized nervous systems. Orientation is anterior to the left and ventral down. Scale bars are 100 μm for A and 50 μm for B & C. The region of animals shown in B & C is indicated by the rectangle in A. A, Diagram of major neuron processes in** ***C. elegans.*** **B, Lateral view of a wild type worm carrying a construct expressed throughout the nervous system (F25B3.3 promoter fused to GFP) displaying a normal ventral nerve cord (V) and commissures (arrowhead). F25B3.3 has an expression pattern identical to that of** ***unc-119*** **except that expression begins later in development. C, Lateral view of an** ***unc-119*** **mutant expressing the same construct as the animal in B. Abnormal commissures are evident (arrowheads). (Diagram courtesy of Wayne Materi).**


A
Pharynx
Nerve Ring
Pharynx Terminal Bulb
Dorsal Nerve Cord
Commissure
Amphid Sensilla
Ventral Nerve Cord


B
C

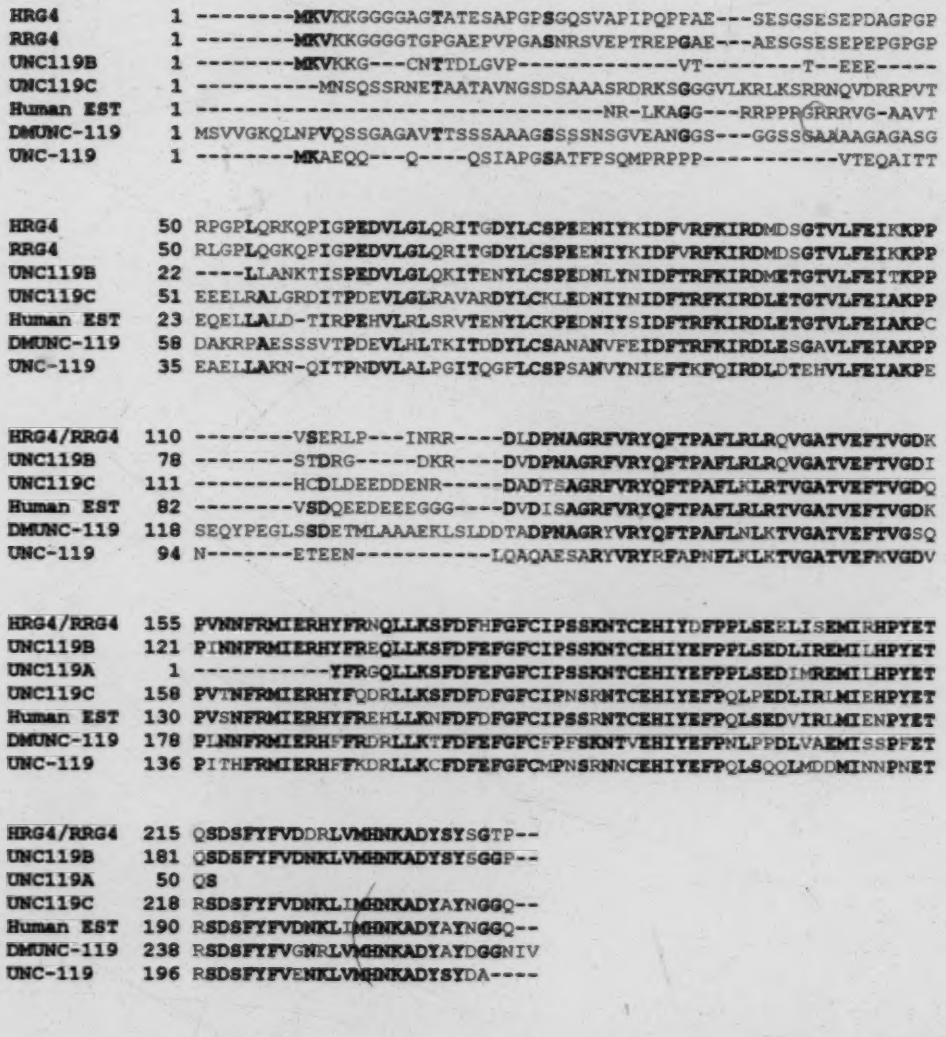


Figure 2 Amino acid alignment of UNC-119 Family Members. Black text denotes identity in three or more sequences, grey text denotes similarity or non-identity. HRG4 and RRG4 sequences are combined since they are 100% identical after amino acid 57. Accession numbers are as follows : HRG4 (U40998), RRG4 (U40999), Human EST (N41778), DMUNC-119 (AF119102) and UNC-119 (U32854). Accession numbers have not been assigned to zebrafish UNC119A, UNC119B or UNC119C.

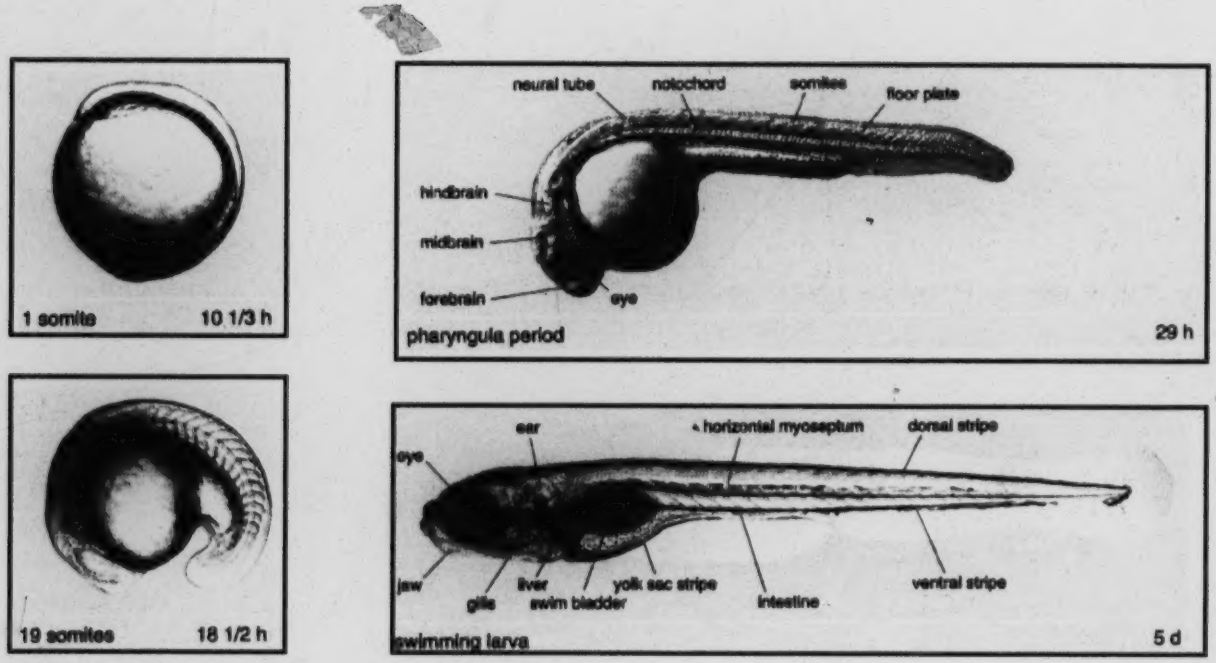
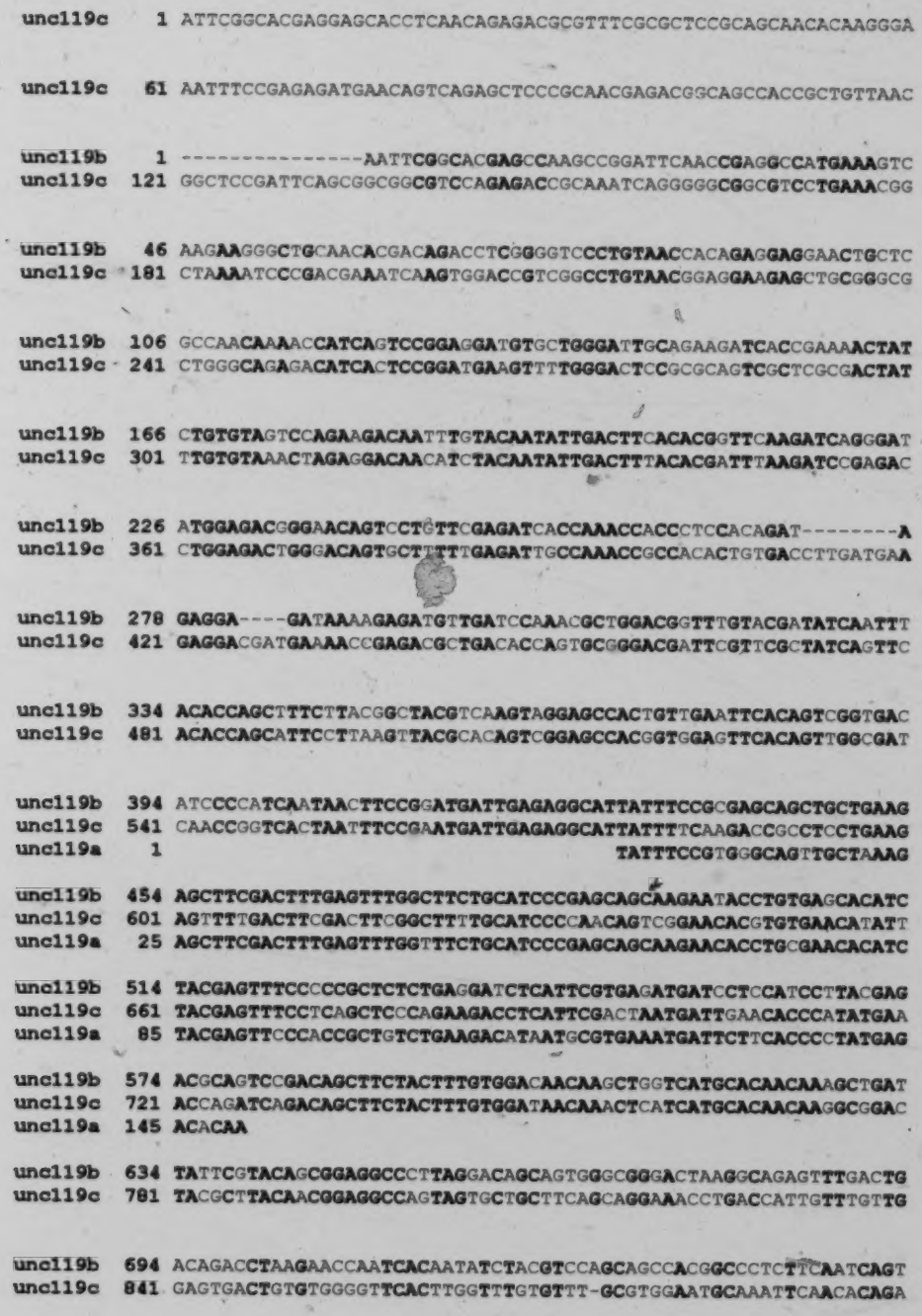


Figure 3. Representative stages of the zebrafish embryo (adapted from Haffter *et al.*, 1996) h = hours post-fertilization d= days post-fertilization

Figure 4 Nucleotide alignment of zebrafish *unc119b*, *unc119c* and *unc119a* (partial) sequences. Black text denotes identical nucleotides in at least two sequences, grey text denotes unmatched nucleotides. The nucleotide sequence of the *unc119a* intron is shown below the alignment.


unc119c 1 ATTCGGCACGAGGAGCACCTCAACAGAGACGCGTTTCGCGCTCCGCAGCAACACAAGGGA
unc119c 61 AATTTCCGAGAGATGAACAGTCAGAGCTCCCGCAACGAGACGGCAGCCACCGCTGTTAAC
unc119b 1 ----------AATTCGGCACGAGCCAAGCCGGATTCAACCGAGGCCATGAAAGTC
unc119c 121 GGCTCCGATTCAGCGGCGGCGTCCAGAGACCGCAAATCAGGGGGCGGCGTCCTGAAACGG
unc119b 46 AAGAAGGGCTGCAACACGACAGACCTCGGGGTCCCTGTAACCACAGAGGAGGAACTGCTC
unc119c 181 CTAAAATCCCGACGAAATCAAGTGGACCGTCGGCCTGTAACGGAGGAAGAGCTGCGGGCG
unc119b 106 GCCAACAAAACCATCAGTCCGGAGGATGTGCTGGGATTGCAGAAGATCACCGAAAACTAT
unc119c 241 CTGGGCAGAGACATCACTCCGGATGAAGTTTTGGGACTCCGCGCAGTCGCTCGCGACTAT
unc119b 166 CTGTGTAGTCCAGAAGACAATTTGTACAATATTGACTTCACACGGTTCAAGATCAGGGAT
unc119c 301 TTGTGTAAACTAGAGGACAACATCTACAATATTGACTTTACACGATTTAAGATCCGAGAC
unc119b 226 ATGGAGACGGGAACAGTCCTGTTCGAGATCACCAAACCACCCTCCACAGAT--------A
unc119c 361 CTGGAGACTGGGACAGTGCTTTTTGAGATTGCCAAACCGCCACACTGTGACCTTGATGAA
unc119b 278 GAGGA----GATAAAAGAGATGTTGATCCAAACGCTGGACGGTTTGTACGATATCAATTT
unc119c 421 GAGGACGATGAAAACCGAGACGCTGACACCAGTGCGGGACGATTCGTTCGCTATCAGTTC
unc119b 334 ACACCAGCTTTCTTACGGCTACGTCAAGTAGGAGCCACTGTTGAATTCACAGTCGGTGAC
unc119c 481 ACACCAGCATTCCTTAAGTTACGCACAGTCGGAGCCACGGTGGAGTTCACAGTTGGCGAT
unc119b 394 ATCCCCATCAATAACTTCCGGATGATTGAGAGGCATTATTTCCGCGAGCAGCTGCTGAAG
unc119c 541 CAACCGGTCACTAATTTCCGAATGATTGAGAGGCATTATTTTCAAGACCGCCTCCTGAAG
unc119a 1 TATTTCCGTGGGCAGTTGCTAAAG
unc119b 454 AGCTTCGACTTTGAGTTTGGCTTCTGCATCCCGAGCAGCAAGAATACCTGTGAGCACATC
unc119c 601 AGTTTTGACTTCGACTTCGGCTTTTGCATCCCCAACAGTCGGAACACGTGTGAACATATT
unc119a 25 AGCTTCGACTTTGAGTTTGGTTTCTGCATCCCGAGCAGCAAGAACACCTGCGAACACATC
unc119b 514 TACGAGTTTCCCCCGCTCTCTGAGGATCTCATTCGTGAGATGATCCTCCATCCTTACGAG
unc119c 661 TACGAGTTTCCTCAGCTCCCAGAAGACCTCATTCGACTAATGATTGAACACCCATATGAA
unc119a 85 TACGAGTTCCCACCGCTGTCTGAAGACATAATGCGTGAAATGATTCTTCACCCCTATGAG
unc119b 574 ACGCAGTCCGACAGCTTCTACTTTGTGGACAACAAGCTGGTCATGCACAACAAAGCTGAT
unc119c 721 ACCAGATCAGACAGCTTCTACTTTGTGGATAACAAACTCATCATGCACAACAAGGCGGAC
unc119a 145 ACACAA
unc119b 634 TATTCGTACAGCGGAGGCCCTTAGGACAGCAGTGGGCGGGACTAAGGCAGAGTTTGACTG
unc119c 781 TACGCTTACAACGGAGGCCAGTAGTGCTGCTTCAGCAGGAAACCTGACCATTGTTTGTTG
unc119b 694 ACAGACCTAAGAACCAATCACAATATCTACGTCCAGCAGCCACGGCCCTCTTCAATCAGT
unc119c 841 GAGTGACTGTGTGGGGTTCACTTGGTTTGTGTTT-GCGTGGAATGCAAATTCAACACAGA

```
unc119b   754 GTCATAGGCGAGCGGCTGCAGTGCGTCATTCCTGTTTTGGAGATCTAGAGAGTCACACCA
unc119c   900 CTCTTA--CTACCAACAAAAACAAAACTATACACGTTGACACCAGCACACCATCATGGCA

unc119b   814 ATAAGGTCTTGTCCGACACGCACACATATCCTATCCTG--------ATTTCACACACTGC
unc119c   958 CCATCGACCCACCCAACTGGAAATAATGACCATTACTCTATGATGAAGTTCCTGCATTCC

unc119b   866 CATTCACGTCAG---CAAAAACACAAACTTAAGCATTTATTTTGAAGCGTACCCCAATT-
unc119c  1018 ACTCTACTTGTGGGTCTGGCCAAAAAAATGGAGCATTTCTCCTTCGACTAGTCGGAAGCG

unc119b   922 ATGAGCTAACACGTCCCATAGTTTGTATGGGCAAAGCACTGTAACGTGAAT--GGTACTT
unc119c  1078 AAGAATTCTGTCATCCTGCTTTTAGGGTGACTACTATAGAGGAGTTTGGTTTTGTAACTT

unc119b   980 AGGTTTTACTATTCATTGAGTGAGTGCGGTGTGTGTGAGATTTGATTCAATGATTGCAAG
unc119c  1138 GCGTTGTCTTCTTGCACCATTAACCTTTTGTCTTGAATTGTTTTTTTGAAGTCGTGTTAC

unc119b  1040 TGTGTGTGTGTGTGAGATGACGCAGATATGACCGTTATATATTGTTGATGTTGAGTATTT
unc119c  1198 CAAACATAATCTTGAACATCTGGATATTTTGGAATATTATTCAGATGTGCTAAGATAAAG

unc119b  1100 TTTCCCTGTCAGAATAAGCTACATCAAATTTCATACTTGTGAACTTTTCATCCTAAATAG
unc119c  1258 CATTCCTGCCGTAGGTTTGGTCA-CTAATGTGAAGTTGCACAGATTTTGCTGTTTTATTA

unc119b  1160 AAGCTGATTTTATGGTGAGACCTTGACTTAATTGATAGATTTTGTGTTGATTAGATATAT
unc119c  1317 TCAGTCATTAGAGGGAGAGAAAGCTTGATTGGTGCACATCCTCTCTTTGTCAAAGTAAAC

unc119b  1220 TTTGATACGTGAACAAACTCACTTCTGAGATGTCGATTATTTGTGGGAATAAAACAAAAC
unc119c  1377 TCTTAAGATATTTTCAGATCGGTCAGCATGTCCGGTTCATGACGTGTTATTTTTGTCACT

unc119b  1280 TAAAAAAAAAAAAAGAAAA----------------------------------------
unc119c  1437 CTGACCCTAAAACTGATCAAGTTCACGGTGTTTTGTTCAATTACTGTACTGTTTAAAGGT

unc119c  1497 TGGGGTCAGTAAAATGTCTAATGTAGACTCTACTGTTCATCAAGGCTACATTTGATCAAA

unc119c  1557 AACACTGTCAAATATCACTTTTGTGAAATAGTGTTAGAACCTTTTTAAAAATCACAGTTT

unc119c  1617 TTAATCTTTTTTCTGCCATGGGATCAAAAATAAATTCATTTGACTGCTAATCTCAAAAAA

unc119c  1677 AAAAAAAAAAAAAA
```

The *unc119a* intron (sequence below) occurs at position 115 of its nucleotide sequence

```
unc119a     1 GTACGTCGCAGACATTTATTAGCAACTCAGAATCTTATTATGCTGATGTGTTGATTGACA
intron        AATGTGTTTTTTTTTTTCCCCACAG 85
```

Figure 5 Sequence of *unc119b* cDNA and position of primers

Putative start and stop codons are underlined and bolded; the polyadenylation signal is overlined. Primer mismatches arising in primers designed using homologous sequences are shown as bolded nucleotides (e.g. these primers were "guess-primers" designed to obtain additional zebrafish sequence at the beginning of this project).

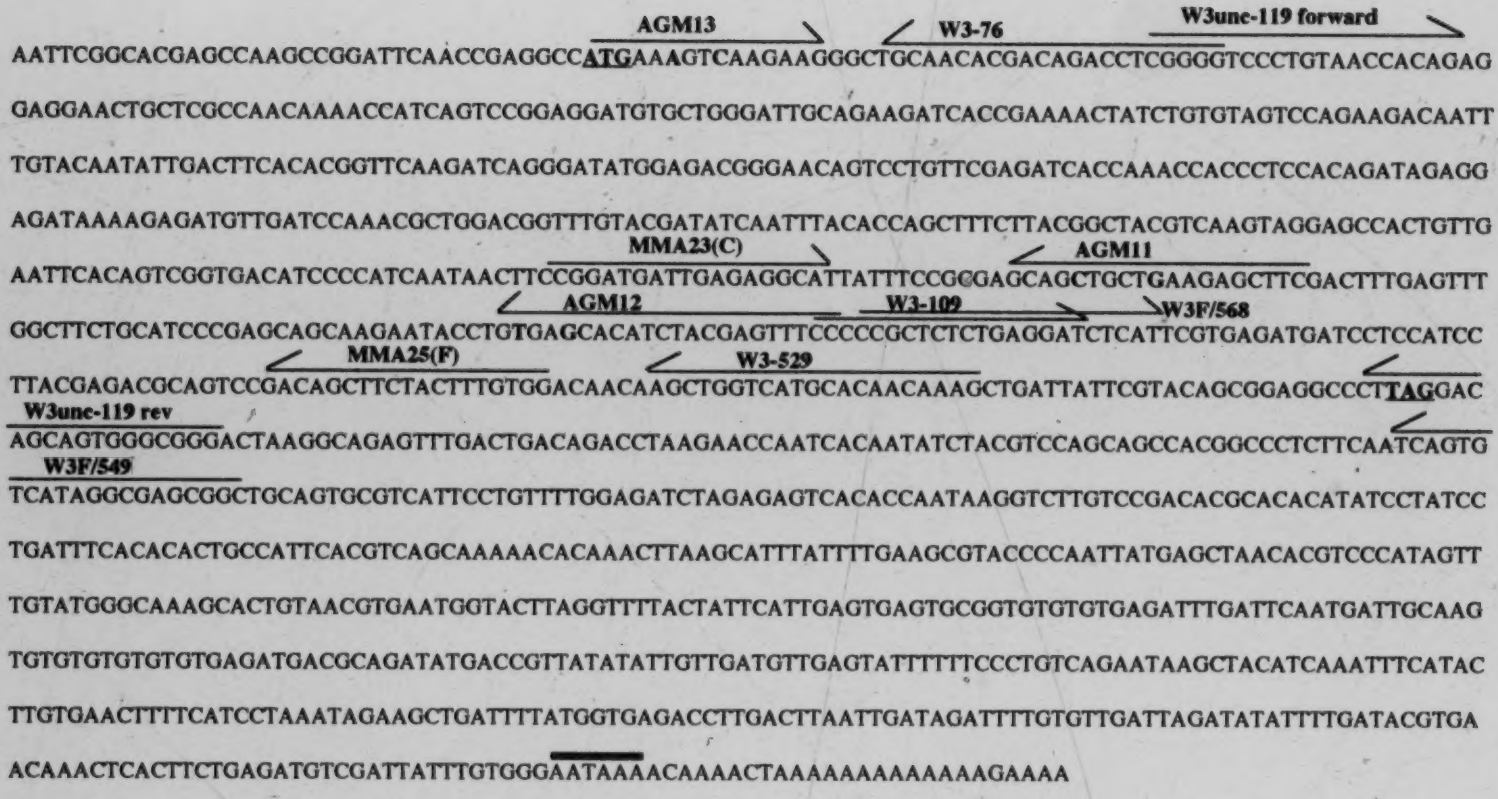

AGM13
W3-76
W3unc-119 forward
ATG
MMA23(C)
AGM11
AGM12
W3-109
W3F/568
MMA25(F)
W3-529
TAG
W3unc-119 rev
W3F/549

Figure 6 Sequence of *unc119c* cDNA and position of primers

Putative start and stop codons are underlined and bolded; the polyadenylation signal is overlined. Primer mismatches arising in primers designed using homologous sequences are shown as bolded nucleotides (e.g. these primers were "guess-primers" designed to obtain additional zebrafish sequence at the beginning of this project).

ATTCGGCACGAGGAGCACCTCAACAGAGACGCGTTTCGCGCTCCGCAGCAACACAAGGGAAATTTCCGAGAGATGAACAGTCAGAGCTCCCGC
AACGAGACGGCAGCCACCGCTGTTAACGGCTCCGATTCAGCGGCGGCGTCCAGAGACCGCAAATCAGGGGGCGGCGTCCTGAAACGGCTAAAA
W%A-215
TCCCGACGAAATCAAGTGGACCGTCGGCCTGTAACGGAGGAAGAGCTGCGGGCGCTGGGCAGAGACATCACTCCGGATGAAGTTTTGGGACTCC
GCGCAGTCGCTCGCGACTATTTGTGTAAACTAGAGGACAACATCTACAATATTGACTTTACACGATTTAAGATCCGAGACCTGGAGACTGGGAC
W5A/360
W5A-459
AGTGCTTTTTGAGATTGCCAAACCGCCACACTGTGACCTTGATGAAGAGGACGATGAAAACCGAGACGCTGACACCAGTGCGGGACGATTCGTT
CGCTATCAGTTCACACCAGCATTCCTTAAGTTACGCACAGTCGGAGCCACGGTGGAGTTCACAGTTGGCGATCAACCGGTCACTAATTTCCGAAT
MMA23(C)
W5-63
GATTGAGAGGCATTATTTTCAAGACCGCCTCCTGAAGAGTTTTGACTTCGACTTCGGCTTTTGCATCCCCAACAGTCGGAACACGTGTGAACATA
AGM12
MMA25(F)
TTTACGAGTTTCCTCAGCTCCCAGAAGACCTCATTCGACTAATGATTGAACACCCATATGAAACCAGATCAGACAGCTTCTACTTTGTGGATAAC
W5A/787
AAACTCATCATGCACAACAAGGCGGACTACGCTTACAACGGAGGCCAGTAGTGCTGCTTCAGCAGGAAACCTGACCATTGTTTGTTGGAGTGAC
TGTGTGGGGTTCACTTGGTTTGTGTTTGCGTGGAATGCAAATTCAACACAGACTCTTACTACCAACAAAAACAAAACTATACACGTTGACACCAG
CACACCATCATGGCACCATCGACCCACCCAACTGGAAATAATGACCATTACTCTATGATGAAGTTCCTGCATTCCACTCTACTTGTGGGTCTGGC
CAAAAAAATGGAGCATTTCTCCTTCGACTAGTCGGAAGCGAAGAATTCTGTCATCCTGCTTTTAGGGTGACTACTATAGAGGAGTTTGGTTTTGT
AACTTGCGTTGTCTTCTTGCACCATTAACCTTTTGTCTTGAATTGTTTTTTTGAAGTCGTGTTACCAAACATAATCTTGAACATCTGGATATTTTGG
AATATTATTCAGATGTGCTAAGATAAAGCATTCCTGCCGTAGGTTTGGTCACTAATGTGAAGTTGCACAGATTTTGCTGTTTTATTATCAGTCATT
AGAGGGAGAGAAAGCTTGATTGGTGCACATCCTCTCTTTGTCAAAGTAAACTCTTAAGATATTTTCAGATCGGTCAGCATGTCCGGTTCATGACG
W5A/215
TGTTATTTTTGTCACTCTGACCCTAAAACTGATCAAGTTCACGGTGTTTTGTTCAATTACTGTACTGTTTAAAGGTTGGGGTCAGTAAAATGTCTA
ATGTAGACTCTACTGTTCATCAAGGCTACATTTGATCAAAAACACTGTCAAATATCACTTTTGTGAAATAGTGTTAGAACCTTTTTAAAAATCAC
AGTTTTTAATCTTTTTTCTGCCATGGGATCAAAAATAAATTCATTTGACTGCTAATCTCAAAAAAAAAAAAAAAAAAAA

Figure 7 Northern analysis of *unc119b* expression in adult zebrafish. A, A blot containing total RNA isolated from adult male (M) or female (F) zebrafish isolated at various timepoints of sacrifice (A= 9:30am, B= 5:30 pm, C= 9:30 pm) to detect possible circadian fluctuations. A photo of the 18S ribosomal RNA present in the original gel serves as the loading control. B, A blot containing a mixture of total RNA from adult male (M) or female (F) zebrafish sacrificed at the same timepoints as described above. Hybridization of this blot with human β-actin (Clontech) at lower stringency (60°C) serves as a loading control. Both blots were probed with the complete 1.3 kilobase cDNA (pDP#*unc119b* insert).

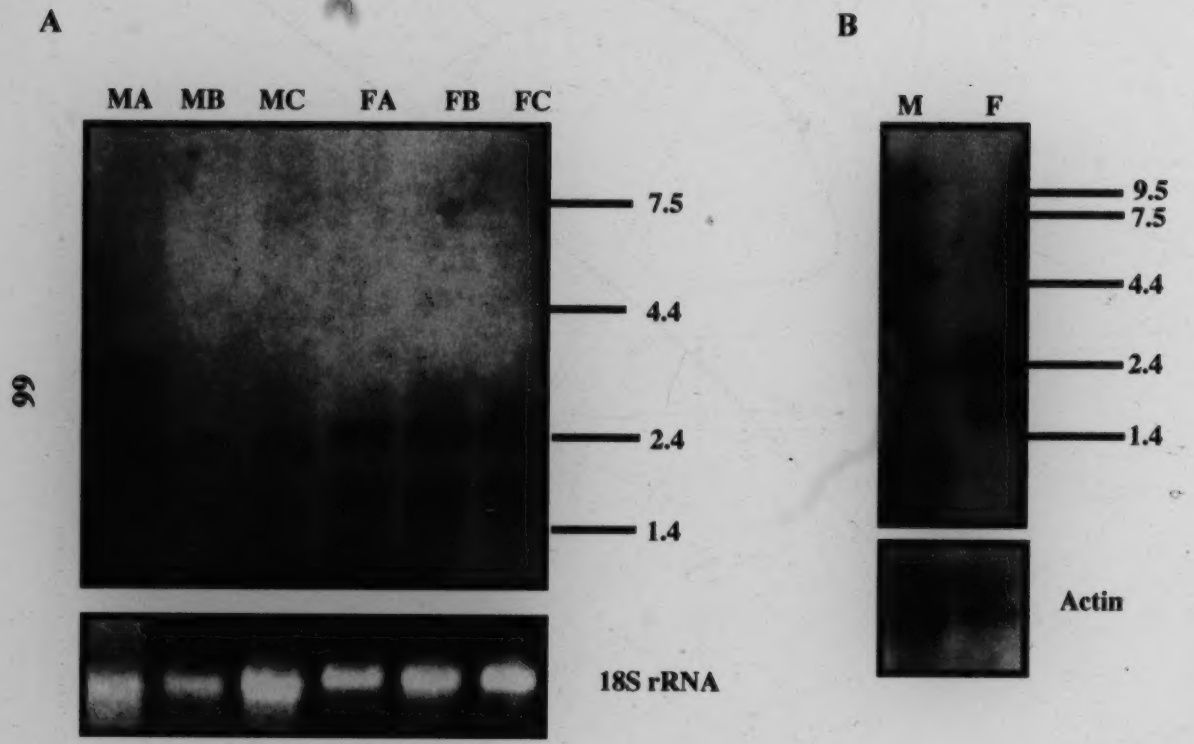

A
MA
MB
MC
FA
FB
FC
7.5
4.4
2.4
1.4
18S rRNA
B
M
F
9.5
7.5
4.4
2.4
1.4
Actin

Figure 8 Northern analysis of *unc119c* expression in adult zebrafish. A, A blot containing total RNA isolated from adult male (M) or female (F) zebrafish isolated at various timepoints of sacrifice (A= 9:30am, B= 5:30 pm, C= 9:30 pm) to detect possible circadian fluctuations. A photo of the 18S ribosomal RNA present in the original gel serves as the loading control. B, A blot containing a mixture of total RNA from adult male (M) or female (F) zebrafish sacrificed at the same timepoints as described above. Hybridization of this blot with human β-actin (Clontech) at lower stringency (60°C) serves as a loading control. Both blots were probed with the complete 1.7 kilobase cDNA (pDP#*unc119c* insert).

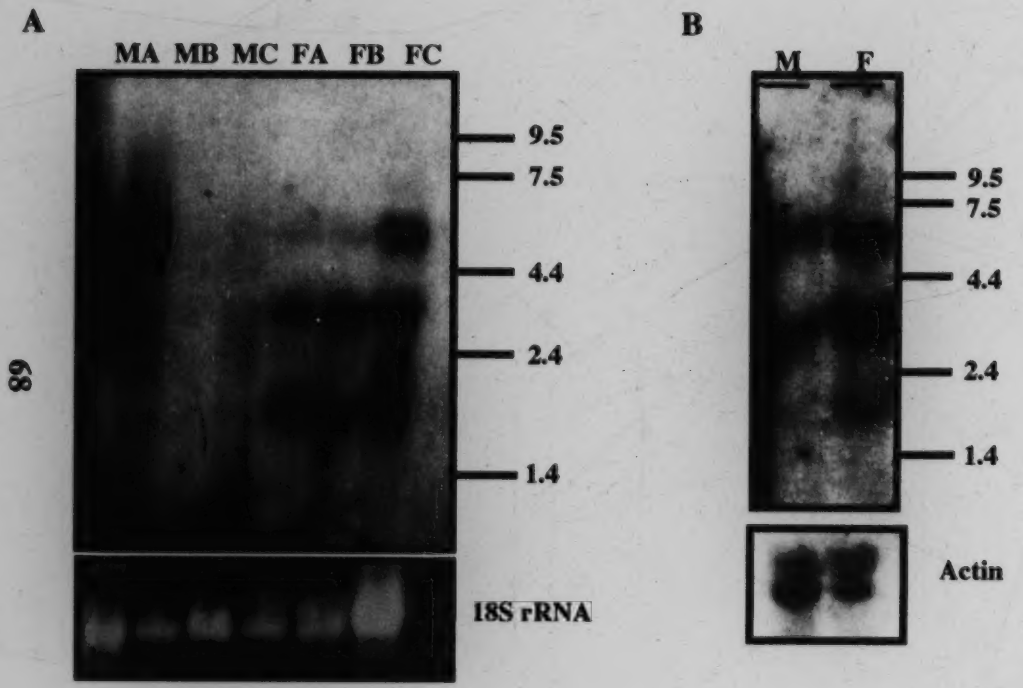
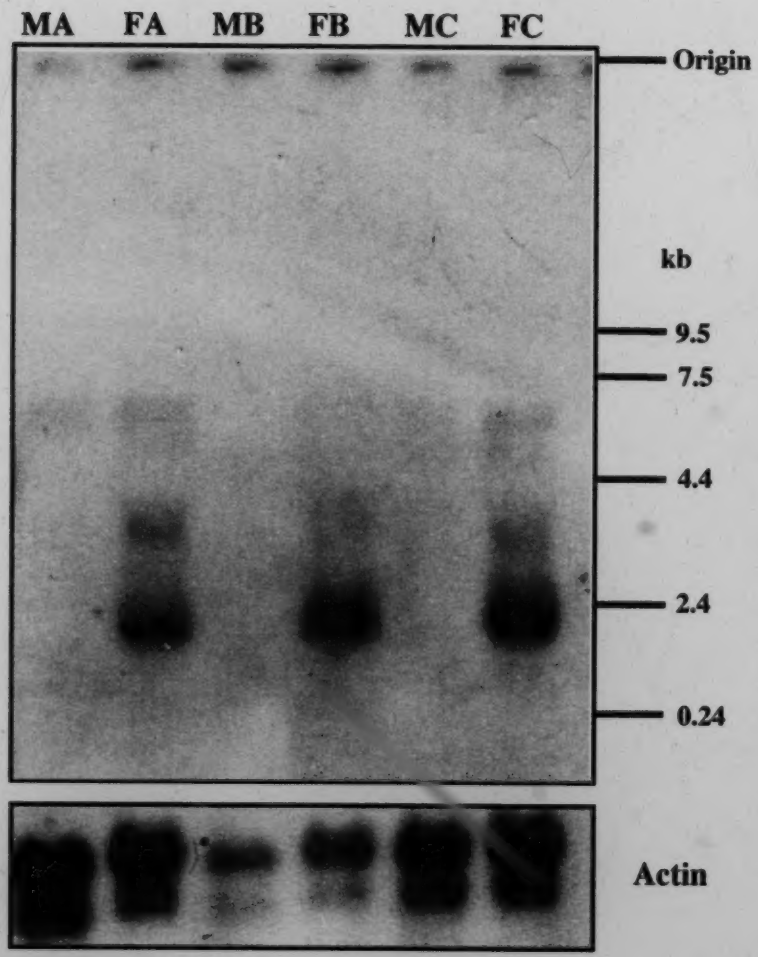

A
MA MB MC FA FB FC
9.5
7.5
4.4
2.4
1.4
18S rRNA
B
M F
9.5
7.5
4.4
2.4
1.4
Actin

Figure 9 Northern analysis of PDP2 expression in adult zebrafish. A blot containing total RNA isolated from adult male (M) or female (F) zebrafish isolated at various timepoints of sacrifice (A= 9:30am, B= 5:30 pm, C= 9:30 pm) to detect possible circadian fluctuations. The blot was probed with a partial 650 basepair PDP2 probe derived from pDP#PSB5 (cloned by Paul Stothard). Hybridization of this blot with human β-actin (Clontech) at lower stringency (60°C) serves as a loading control.


MA
FA
MB
FB
MC
FC
Origin
kb
9.5
7.5
4.4
2.4
0.24
Actin

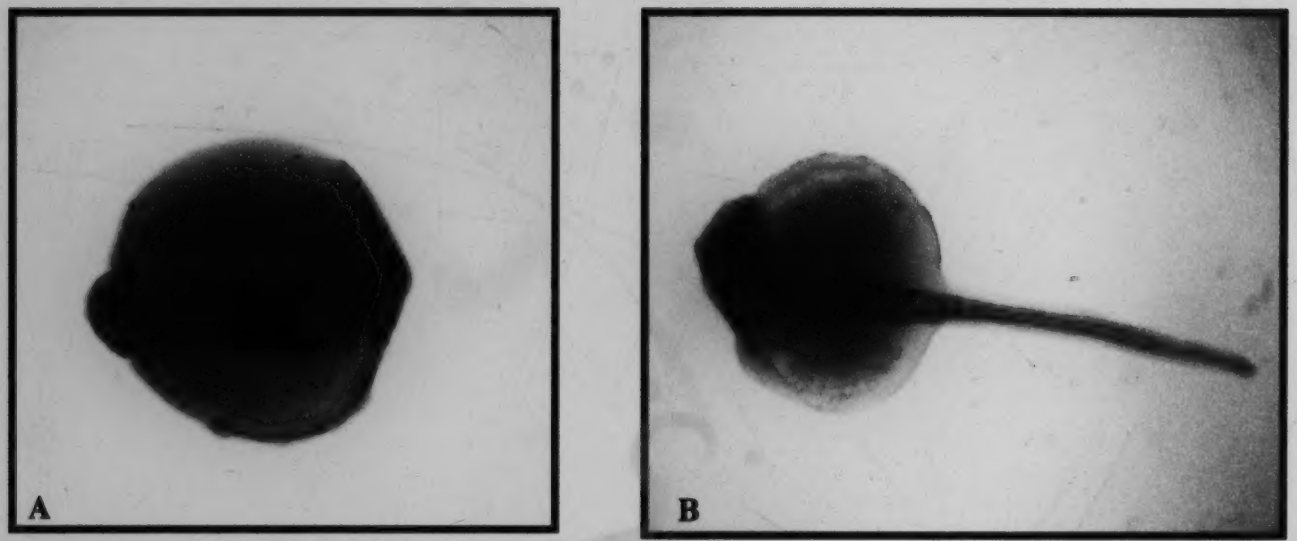


Figure 10 ***In situ*** **hybridization controls. A,** Krox-20 staining of rhombomeres 3 & 5 of the hindbrain in a 13 hpf embryo **B,** Dorsal view of a 23 hpf embryo stained with a somite specific probe. Orientation is anterior to the left. Probes provided by Yilin Yan, Univeristy of Oregon.

Figure 11 *In situ* hybridization with zebrafish *unc119b* and *unc119c* genes. A, 23 hpf embryo stained with an *unc119c* sense control probe. B, 23 hpf embryo stained with an *unc119c* antisense probe. C, 34 hpf embryos stained with an *unc119b* sense (upper left) or antisense probe (lower right). D, Higher magnification of the antisense stained embryo in C. E, 50 hpf embryos stained with an *unc119c* sense (upper left) or antisense probe. F, 61 hpf embryos stained with an *unc119b* sense (upper left) or antisense probe. All pictures were taken using a 5X objective, except the embryo in D for which a 10X objective was used. Probes were DIG-11-UTP labeled inserts of pDP#*unc119b* or pDP#*unc119c*. At this level of analysis *unc119b* and *unc119c* genes appear to have identical expression patterns and therefore, photographs are representative for either staining reaction. Structures are labeled as follows: B' = brain, E' = eye, F' = fin bud and Y = yolk sac.

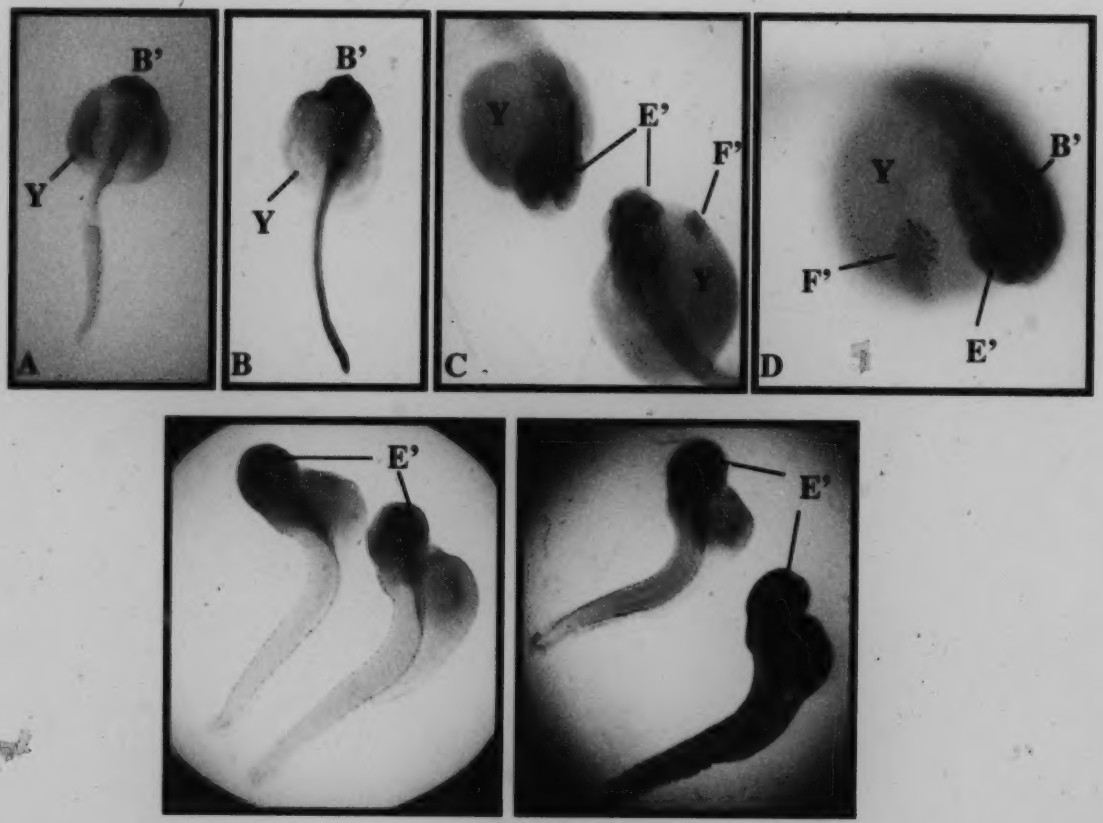

B'
Y
A
B'
Y
B
Y
E'
F'
Y
C
Y
B'
F'
E'
D
E'
E'

Figure 12 *In situ* hybridization with the zebrafish PDP2 gene. A, 23 hpf embryos stained with a sense (upper left) or antisense (lower right) PDP2 probe. B, 24 hpf embryo stained with a PDP2 sense probe. C, 24 hpf embryo stained with a PDP2 antisense probe. D, 34 hpf embryos stained with a sense (left) or antisense (right) PDP2 probe. E, 61 hpf embryos stained with a sense (bottom) or antisense (upper) PDP2 probe. All pictures were taken using a 5X objective. Probes were DIG-11-UTP labeled inserts of pDP#PDP2XhoI#1 (cloned by Paul Stothard). Structures are labeled as follows: B' = brain, E' = eye, F = fin bud and Y = yolk sac.

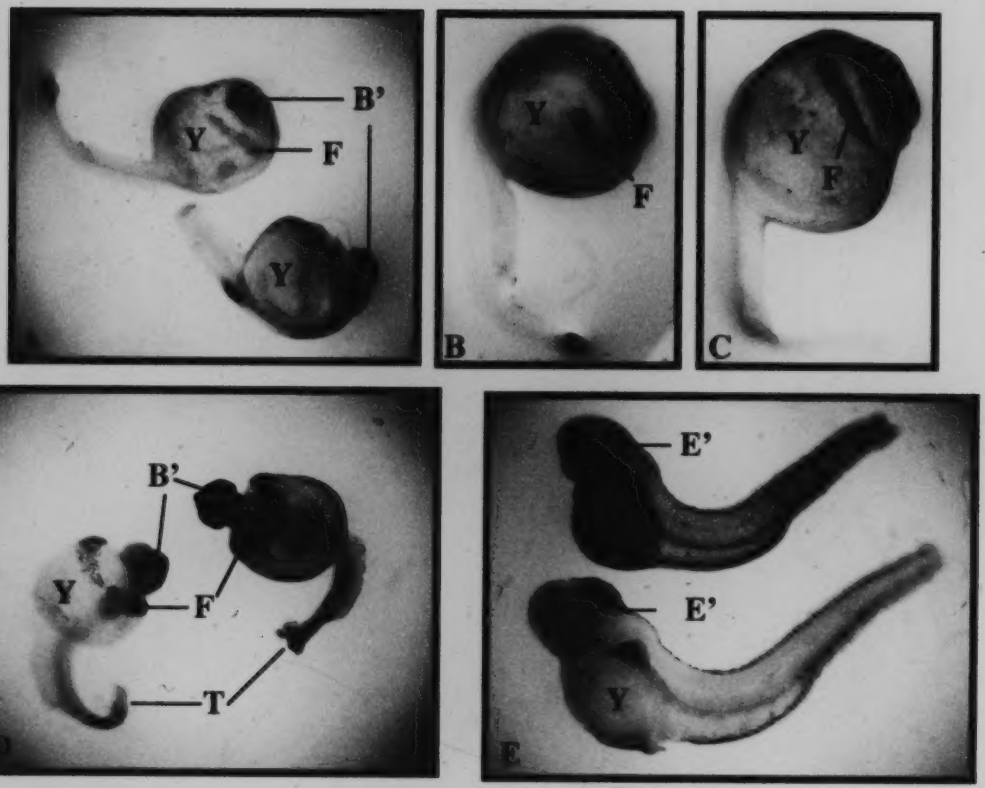

B'
Y
F
Y
B
Y
F
C
Y
F
B'
Y
F
T
D
E'
E'
Y
E

Table 1. A list of plasmids used in this study. Plasmids are listed along with a brief description of features or other helpful information.

Plasmid	Description	Vector/Other Information
pDP#*unc119a*	Partial (238 bp) zf *unc-119* homologue obtained through degenerate PCR using genomic DNA	pBluescript (KS-) Liberate insert with BamHI Product contains an 85 nt intron
pDP#W3unc-119	Partial (150 bp) zf *unc119b* obtained through degenerate PCR using adult cDNA	pGEM-T Insert has PvuII site
pDP#*unc119b*	1.3 kb zf *unc-119* homologue obtained through screening an embryonic zf cDNA library. Contains sequence of pDP#W3unc-119 above	pBluescript Shares the "MKVKKG" sequence that occurs at the start of HRG4/RRG4 May not be full-length
pDP#*unc119c*	1.7 kb zf *unc-119* homologue obtained through screening an embryonic zf cDNA library. Contains sequence of pDP#W5unc-119 below.	pBluescript Full insert liberated with XhoI – NotI digest May not be full-length
pDP#W5unc-119	Partial (150 bp) zf *unc119c* obtained through degenerate PCR using adult cDNA	pGEM-T Insert has NdeI site
pDP#PSB5	Partial (850bp) zf PDP2 homologue obtained through degenerate PCR using adult cDNA as template	pGEM-T Insert digested with PstI/SstII gives 650bp fragment (used as a probe) plus other fragments Paul Stothard used BJM1 & BJM2 degenerate primers to initially amplify PP2Cs from zebrafish; PDP2 was only one of the products obtained
pDP#PDP2XhoI#1	Full length (1.7 kb) zf PDP2 homologue obtained through screening embryonic zf cDNA library	pBluescript Full insert liberated with XhoI – EcoRI digestion Note: The original clone (pDP#PDP2-17) was chimeric; Paul Stothard removed the non-PDP2 sequences and re-ligated the plasmid

Plasmid	Description	Vector/Other Information
Krox-20	Full-length zf Krox-20 cDNA Obtained from Yi-Lin Yan at the University of Oregon	pBluescript Use PstI to linearize & T3 RNA pol to make antisense transcript Expressed strongly in rhombomeres 3 &5 beginning ~11hpf & very faint by 24hpf
Somite	Full-length zf somite-specific cDNA Obtained from Yi-Lin Yan at the University of Oregon	PBluescript Use HindIII to linearize & T3 RNA pol to make antisense transcript Expressed in somite boundaries between ~12hpf – 48 hpf This cDNA has not been fully characterized
Col2a1	Full-length zf collagen cDNA Obtained from Yi-Lin Yan at the University of Oregon	PBluescript Use HindIII to linearize & T3 RNA pol to make antisense transcript Expressed in notochord, floorplate &
zMaxribo	140 bp from 3'UTR of the cDNA Obtained from Nicole Schreiber-Agus at the Albert Einstein College of Medicine, New York	PBluescript Use BamHI to linearize & T7 RNA pol to make antisense Expressed throughout development

Primer name	Primer use/notes	Primer Sequence
MMA23 or C	Degenerate PCR to amplify *unc-119* homologues Note **BamHI** site	5'GACGGATCCGIATGAT(TCA)GAI(AC)GICA(TC)3'
MMA25 or F	As above	5'CTGGGATCCAC(GA)AA(GA)TA(GA)AAI(GC)(AT)(GA)TC3'
AGM13	PCR (unsuccessful) "Guess" primer Note *unc119b* has an A & a C at the bolded positions respectively. *unc119c* does not contain this sequence	5'ATGAAGGTGAAGAAG3' (MKVKK motif)
AGM14	PCR (unsuccessful) "Guess" primer Note *unc119b & unc119c* sequences are too different for this primer to be useful	5'GACTACCTCTGCTCC3' (DYLCS motif)
AGM11	PCR Designed using *unc-119a* sequence; too many mismatches to *unc119c* to be useful; *unc119b* differs at bolded nts	5'CGAAGCTCTTTAGCAACTGC3'
AGM12	PCR Designed using *unc-119a* sequence; *unc119b* differs at bolded nts & *unc119c* differs at underlined nts	5'GGAACTCGTAGATGTGTTCG3'
W5-63	3'RACE for *unc119c*	5'TTGCATCCCCAACAGTCG3'

Primer name	Primer use/notes	Primer Sequence
W3-109	3'RACE for *unc119b*	5'CCCCCGCTCTCTGAGGAT3'
W5A/215	Sequencing *unc119c* cDNA clone	5'CCCCAACCTTTAAACAGTACA3'
W5A/360	As above	5'CCGCCACACTGTGACCTTGAT3'
W5A/787	As above	5'AAGGCGGACTACGCTTACAAC3'
W3F/549	Sequencing *unc119b* cDNA clone	5'GCCGCTCGCCTATGACACTGA3'
W3F/568	As above	5'CCGCTCTCTGAGGATCTTCAT3'
Ribo forward	Control for PDP2 RT-PCR on single zebrafish embryos	5'CGGGCACACCACAATGCGCTGGAG3'
Ribo reverse	As above	5'CGGCTCGGACCCGGAGCTGGAATC3'
W3unc-119 forward	PCR/RT-PCR	5'CGGGGTCCCTGTAACCACAGA3'
W3unc-119 reverse	PCR/RT-PCR	5'CCCGCCCACTGCTGTCCTAAG3'
W%A-215	5'RACE for *unc119c*	5'GGCCGACGGTCCACTTGATTT3'
W5A-459	As above	5'TCCCGCACTGGTGTCAGC3'
W3-76	5'RACE for *unc119b*	5'CCCCGAGGTCTGTCGTGTTGC3'
W3-529	As above	5'GCGGGGGGAAACTCGTAGATGT3'.
BJM1	Degenerate PCR to amplify PP2Cs (PDP2 isolated by Paul Stothard)	5'G(GC)IGTIT(AT)(TC)GA(TC)GGICA(TC)G(GC)IGG3'
BJM2	As above	5'AC(AG)TCCCA(AGT)AIICC(AG)TC(GA)CAIGC3'

Table 2. A list of DNA primers used in this study

Appendix

ANB	- anterior neural boundary organizer
BMP	- bone morphogenic protein
cDNA	- copy DNA, reverse transcribed from mRNA
DIC	- differential interference contrast
dpf	- days post fertilization
ES cells	- embryonic stem cells
EST	- expressed sequence tag
GFP	- green fluorescent protein
hpf	- hours post fertilization
HRG4	- human retinal gene 4
HSN	- hermaphrodite specific neurons
PCR	- polymerase chain reaction
PDP2	-pyruvate dehydrogenase phosphatase 2
PND	- post-natal day
RRG4	- rat retinal gene 4
RT-PCR	- reverse transcriptase polymerase chain reaction
TGF-β	- transforming growth factor beta
VNC	- ventral nerve cord

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